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THE UNIVERSITY OF ALBERTA

STEREOSELECTIVITY IN DRUG METABOLISM, ANALYSIS AND PHARMACOKINETICS

by

© NIKHILESH NIHALA SINGH

A THESIS
SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled STEREOSELECTIVITY IN DRUG METABOLISM, ANALYSIS AND PHARMACOKINETICS submitted by NIKHILESH NIHALA SINGH in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY in PHARMACEUTICAL SCIENCES.

F.M. Pasutto
Supervisor

R.T. Coutts

Glen B. Baker

Fakhreddin Jamali

Walle
External Examiner

Date....August 12, 1986............

to my parents

ABSTRACT

Several aspects of metabolism, analysis and basic pharmacokinetics were investigated from the common perspective of stereoselectivity in biological systems.

Studies were initiated using microorganisms as the basic metabolic units. The fungus Cunninghamella echinulata was selected for (1) the stereospecific oxidation of sulfur in a β-lactam substrate, the methyl ester of 7-[(phenoxy)acetamido]desacetylcephalosporanic acid (CS) and, (2) the regioselective p-hydroxylation of 1-phenoxy-3-(isopropylamino)-2-propanol (PP) to yield the β-agonist prenalterol. The fermentations were scaled up to obtain preparative quantities of the metabolites CS and PP and their structures were confirmed by standard physical methods. The microorganism-mediated metabolic reactions proceeded through capacity limited pathways since the rates of substrate disappearance were linear only at lower concentrations.

A general method was utilized for the stereospecific gas chromatographic (GC) analysis of α-methylbenzeneethanamine (amphetamine) and related analogs. The N-trifluoroacetyl-L-prolyl chloride, amphetamine diastereoisomers were resolved on a cross-linked dimethylsilicone capillary column. The method was then applied to a study of the stereoselective distribution of R- and S-fenfluramine in rat brain and liver after intraperitoneal administration of racemic fenfluramine. It was observed that the relatively inactive R-isomer was preferentially N-dealkylated to norfenfluramine. The proportions of R- and S-fenfluramine and R- and S-norfenfluramine were similar in both tissues.

A novel stereospecific GC method was developed for resolving enantiomers of the arylalkanoic non-steroidal antiinflammatory drugs (NSAIDs) ibuprofen, ketoprofen, naproxen, fenoprofen, flurbiprofen, pirprofen, cicloprofen, tiaprofenic acid and etodolic acid. The NSAIDs were reacted with optically pure S-(+)- or R-(-)-amphetamine and resolved on an achiral dimethylsilicone capillary column. The method was utilized for determining the stereoselective disposition of ibuprofen, tiaprofenic acid and etodolic acid enantiomers in humans. Ibuprofen (plasma AUC of eutomer greater than the distomer) and etodolic acid (plasma AUC of eutomer less than distomer) exhibited marked stereoselectivity while the disposition of tiaprofenic acid enantiomers was non-stereoslective. Three urinary metabolites of etodolic acid were observed; these were identified as 7-hydroxy-, 8,9-dehydrogenated- and N-methyl-derivatives of etodolac.

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ABBREVIATIONS

AE
affinity of a eutomer

AITC
2,3,4-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate

AMP
R-(-)- or S-(+)-amphetamine

APA
2-arylpropionic acid

AUC
area under a plasma concentration-time curve

b
quotient of eudismic affinity

Co
concentration at time 0

Co*
y-(concentration) axis intercept of the log-linear phase of a concentration-time curve

CDA
chiral derivatizing reagent

CDI
1,1'-carbonyldiimidazole

CL
clearance

Cmax
maximum concentration

CMP
chiral mobile phase

CoA
coenzyme A

Concn
concentration

CRF
concentration of prenalterol remaining to be formed

CS
7-[(phenoxy)acetamido]desacetylcephalosporanic acid, methyl ester

CSO
R-sulfoxide of CS

CSP
chiral stationary phase

CXD
cyclodextrin

DANE
(-)-1-(4-dimethylamino-1-naphthyl)ethylamine

DEHP
di-(2-ethylhexyl)phosphate

DIP-MS
direct inlet probe mass spectrometry

EI
eudismic index

ET
etodolic acid (etodolac)

F
fraction absorbed

FA
fluoroamphetamine

FID
flame ionization detector

GC
gas chromatograph(y)

GC/MS
GC/mass spectrometry

GITC
2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate

HETP
height equivalent to theoretical plates

HPLC
high performance liquid chromatograph(y)

IP
ibuprofen

KE
disappearance rate constant for a microbial system

KF
formation rate constant for a microbial system

Kmax
concentration at which the rate of a process is one-half its theoretical maximum rate

L
litre

L'
length in mm

LC/MS
liquid chromatograph/mass spectrometer

LEC
ligand exchange chromatography

M^4
microbial models of mammalian metabol

MBA
R-(+)- or S-(-)-α-methylbenzylamine

mg
milligrams

MBI
α-methylbenzyl isocyanate

MPTA
(-)-α-methoxy-α-(trifluoromethyl)phenylacetic acid

MPTA-Cl
(-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride

MS
mass spectrometer

n
number of theoretical plates

NPD
nitrogen phosphorus detector

NPSP-Cl
4-nitrophenylsulfonyl-S-prolyl chloride

NSAID
non-steroidal antiinflammatory drug(s)

P
prenalterol

p.o.
per os

PEIC
R-(+)- or S-(-)-1-phenylethyl isocyanate

7-PhADCA
7-phenylacetamido-3-deacetoxycephalosporanic acid

PhOADCA
7-phenoxyacetamido-3-deacetoxycephalosporanic acid

PP
pre-prenalterol

T1/2
half-life

TA
tiaprofenic acid

TFAA
trifluoroacetic anhydride

Tmax
time at Cmax

N-TPC
N-trifluoroacetyl-L-prolyl chloride

Vd
volume of distribution

Vmax
maximum rate of the process

Wb
peak width at half peak height (mm)

1.0.0.0.0.0 INTRODUCTION

1.1.0.0.0.0 GENERAL REMARKS

An intriguing aspect of biologically active agents is the discriminatory capacity with respect to their molecular sites of action, more specifically the receptors. This affinity between the drug and the site of binding on the receptor molecule is based upon chemical complimentarity. Apart from the physicochemical affinity or disaffinity of the groups involved in a drug-receptor interaction, the spatial configuration of these groups plays an important role in the process of complimentarity. This role becomes more critical if the drug involved has an asymmetric centre (usually carbon, but also nitrogen, phosphorus or sulfur) thereby leading to the phenomenon of stereoselectivity in the drug-receptor interaction.

Stereoselectivity is the extent to which enzymes or other macromolecular structures (the receptors) exhibit affinity towards one molecule of a pair of stereoisomers in comparison with, and in contrast to, the other molecule (Rauws, 1983). Stereoselective affinity is therefore only apparent if the drug is present as a mixture of stereoisomers. Although several isomeric relationships between molecules are known, further comments are restricted primarily to enantiomers in which the four valencies of a carbon atom are occupied by four different groups (i.e., an asymmetric carbon). These enantiomers are characterized by their non-superimposable mirror image relationship. The drug having an asymmetric carbon or centre in its molecular skeleton will be present as a 50:50 mixture of enantiomers (racemate). Depending upon their individual ability to rotate the plane of polarized light, one isomer is identified as the (+)-, d-, or dextrorotatory enantiomer while the other isomer is the (-)-, l-, or levorotatory enantiomer.

Stereochemistry is inherent in biological processes. Receptors and enzymes are entities understood to be composed of proteins or lipoprotein components that are integrated into the membrane structure (Lehmann, 1976). The native asymmetric properties of the unit components of the enzyme or receptor confer stereoselectivity in drug action. An interaction between the enantiomers of the racemic drug and the biological entity, will result in the formation of a pair of diastereoisomers which now contain at least two asymmetric centres. Unlike enantiomers, diastereoisomers will have different physicochemical properties. Several types of interaction between a drug and its receptor are possible; these include hydrophobic, ionic, dipole-dipole, and hydrogen bonding forces (Ariens, 1983). The nature of various substituent groups in the vicinity of the asymmetric centre may significantly influence these forces of interaction thus making stereoselectivity an even more likely phenomenon.

Stereoselectivity, or more specifically enantioselectivity, has been expressed in a mathematical form, proposed by Lehman (1982). In this nomenclature the enantiomer with the more pronounced receptor affinity or biological activity is described as the 'eutomer' while the enantiomer with lower affinity or activity is known as the 'distomer'. The ratio of activity of the eutomer to distomer is expressed as the 'eudismic ratio' and differences in the logarithmic affinities of eutomer to distomer is described as the 'eudismic index'. The relationship between the eudismic index and the eutomeric affinity or activity is defined by the equation of a straight line:

$$EI = a + b\log AE$$

where EI = eudismic index

b = eudismic affinity quotient

AE = affinity of the eutomer

The potency of a racemate is often expressed as

Pfieffer's rule (Pfieffer, 1956). Within the framework of this discussion, the terms affinity and activity are not synonymous. For example, as a result of differences in affinity, one enantiomer may exhibit potent agonist properties while the other is a weak agonist (e.g. muscarine isomers) (Dahlbom, 1983). However, it is also possible that enantiomers may have the same degree of affinity but exhibit an agonist-antagonist relationship (e.g. isoproterenol isomers)(Portoghese, 1970). On the basis of these considerations it has been suggested that racemic drugs are 'pseudo-hybrid' products (Ariens, 1984).

Enantioselectivity in a drug-receptor (enzyme) interaction may result in the stereoselective absorption, distribution, metabolism and excretion of drugs. Each of these processes, alone or in conjunction with others, can significantly alter the pharmacokinetics of a xenobiotic. Therefore enantiomer-induced selectivity during the in-vivo processing of a racemate may lead to pharmacokinetic stereoselectivity. It is of interest to note that there is no apparent association between pharmacological and pharmacokinetic stereoselectivity. For example, even though anorectic activity primarily resides with the (S)-isomer of fenfluramine, the pharmacokinetic disposition of the isomers is similar in humans (Caccia et al., 1982).

Conclusive determination of pharmacokinetic stereoselectivity is dependent upon the analytical technique employed in monitoring the time course of the biological levels of the xenobiotic. Pharmacokinetic data generated by analytical procedures that measure total drug concentration (eutomer plus distomer), rather than individual enantiomers, may result in misleading, if not incorrect, information (Ariens, 1984). It is therefore important that such studies be based on analytical techniques which are reliable not only for their precision, accuracy and reproducibility but also for their ability to quantify each enantiomer independently.

Thus, an appreciation for the importance of enantioselectivity in drug action and the need for development of gas chromatographic methods capable of separating and quantifying enantiomers formed the basis of the investigations described in this thesis.

1.2.0.0.0.0 LITERATURE SURVEY

1.2.1.0.0.0 RESOLUTION OF ENANTIOMERS BY CHROMATOGRAPHY

The selection of a chromatographic method from the various available techniques is frequently made for practical reasons. The requirement of large sample size and tedious work-up procedures makes the polarimetric method unsuitable for metabolic studies. Similarly, circular dichroism, Cotton-curve effects, X-ray crystallography and nuclear magnetic resonance are of preparative and qualitative significance only. Stereospecific radioimmunoassays do not have general applicability and tend to be costly. Most drug metabolism and analytical laboratories are equipped with a gas chromatograph (GC) and/or a high performance liquid chromatograph (HPLC). The sensitivity and superior resolving capabilities associated with chromatographic methods make them ideal for stereospecific analyses.

The quantitative measurement of separation between the chromatographic (GC) peaks is expressed as α (separation factor, resolution factor or relative volatility)(Rose et al., 1966). Mathematically,

$$\alpha = \frac{RT_2 - RT_0}{RT_1 - RT_0}.$$

where RT^2 and RT^1 are retention times for the first and second components respectively and RT_a the retention time for the inert gas. The term α can also be related to the gas-liquid partition coefficients or free energy differences of the diastereoisomers in case of an isothermal run (Schurig, 1984), to enthalpy and entropy during a programmed run (Beitler and Feibush, 1976), and to vapour pressure and activity coefficient of the selectand (the racemic mixture) during direct resolution (Lochmuller and Souter, 1975).

In the ensuing discussion the terms optical resolution, enantiomer separation or resolution, and chiral resolution or separation have been used synonymously and denote the chromatographic resolution of enantiomers. Even though chiral separations have been achieved using various chromatographic techniques (Gil-Av et al., 1964; Koenig et al., 1970; Frank et al., 1978a; Armstrong and DeMond, 1984, Francotte et al., 1985b), for the purposes of this thesis, emphasis has been placed on GC, and, when appropriate, on HPLC as well.

1.2.1.1.0.0 **METHODS IN CHROMATOGRAPHIC RESOLUTION**

Chiral resolution of a racemate can be achieved by two chromatographic methods, namely:

i) the indirect method (Gil-Av et al., 1964; Pollock et al., 1965; Pollock, 1966), and

ii) the direct method (Koenig et al., 1970; Nakaparksin et al., 1970)

1.2.1.1.1.0 THE INDIRECT METHOD OF CHROMATOGRAPHIC ENANTIOMER RESOLUTION

This method involves conversion of the enantiomers into diastereoisomers, using a suitable auxiliary chiral reagent, followed by chromatography on an achiral phase. The first review dealing with the resolution of diastereoisomers by GC appeared in 1974 (Gil-Av and Nurok, 1974). The chemical events involved in the formation of diastereoisomers may be represented by

$$A_1A_2 + B^* \longrightarrow A_1B^* + A_2B^*$$

where A_1A_2 is the racemate containing the two enantiomers A_1 and A_2

B^* is the chiral auxiliary reagent of high or absolute optical purity, and

A_1B^* and A_2B^* represent a pair of diastereoisomers formed by the chemical reaction.

Except for their ability to rotate a plane of polarized light in opposite directions, enantiomers have identical physical and chemical properties (Morrison, 1983); the separation of a pair of enantiomers in an achiral environment is therefore impossible. As a consequence, reports claiming chiral resolution under achiral circumstances (Sczepaniak and Ciszewska, 1982, 1984; Kundy and Crooks, 1983; Charles and Gil-Av, 1984) should be interpreted cautiously (Schurig, 1984; Davankov and Kurganov, 1983; Bayer, 1984). After the introduction of a second asymmetric centre, present in the auxiliary chiral reagent, the resulting pair of diastereoisomers are theoretically separable because they have different physicochemical properties.

1.2.1.1.1.1 DIASTEREOISOMERIC DERIVATIVES-STRUCTURAL REQUIREMENTS

Diastereoisomer formation is not the only requirement for the resolution of enantiomers, nor can it be assumed that diastereoisomers are always separable. Several structural features appear to play a significant role in determining the degree of resolution. These features are discussed in the following Sections 1.2.1.1.1.1.a to 1.2.1.1.1.1.c.

1.2.1.1.1.1.a STRUCTURAL RIGIDITY AND DIASTEREOISOMER RESOLUTION

Westley et al. (1968) and Westley and Halpern (1968) determined the effect of various conformational changes on the separation of diastereoisomeric amides and esters. With ester derivatives, represented by Figure 1, it was observed that optimal resolutions were obtained by the combination of a small and a branched alkyl group, e.g. R=methyl and R'=isopropyl groups respectively. A further improvement in separation was achieved when one of the asymmetric centres was part of a ring system. A similar situation was observed with structurally related amides (Karger et al., 1967). The common theme in these studies is that conformational immobility (by branching or rings) assists in the creation of an asymmetric environment around the ester or amide function. This asymmetry provides for nonequivalent accessibility of the functional group of each of the diastereoisomeric pair for interactions with the stationary phase.

1.2.1.1.1.1.b EFFECT OF CHIRAL CENTRE INTERDISTANCES ON THE RESOLUTION OF DIASTEREOISOMERS

A systematic movement of the asymmetric centres away from each other, while keeping the molecular weight constant, may lead to a loss in resolution. Rose et al. (1966) investigated a series of diastereoisomeric esters to determine the effect of chiral centre interdistances on resolution. They concluded that the distance between chiral centres and conformational immobility are, in fact, interrelated parameters. While investigating the resolution characteristics of diastereoisomeric ester (lactate) derivatives of cyanohydrins, Julia and Sans (1979) confirmed the observations made by Rose and co-workers (1966). However, in the studies described above, the systematic displacement of the chiral centre away from the ester linkage resulted only in poor resolution rather than its complete loss. The mechanism was rationalized using the differential interaction and conformational immobility theory originally proposed by Karger in 1967. Replacement of 'H' by a 'CN' group increases the differences in properties between pairs of diastereoisomers and thus substantially overcomes the loss of immobility.

From the preceeding discussion it is apparent that the resolution of diastereoisomers is influenced by several steric factors; substituents which confer conformational immobility, either by virtue of their cyclic nature or steric bulk, improve resolution. Studies on diastereoisomeric amide derivatives of various amino acids have resulted in the same conclusion (Iwase and Murai, 1974a, 1974b; Iwase, 1974a, 1974b).

It is unfortunate that only a few studies with various other functional groups have been conducted. This would have led to an unequivocal confirmation (or disproval) of the above postulates. Another aspect which has not been investigated is the comparative behaviour of various functional group linkages for analogous derivatives. This is important as the postulated forces of bonding between various linkages and chromatographic stationary phases will not be identical. Any benefits obtained through bulk asymmetry and subsequent nonequivalent accessibility would be immediately lost due to a weak interaction between the group linking the isomers and the stationary phase. The 1-naphthalenemethylamide derivative of ibuprofen was resolvable on a chiral column whereas the 1-naphthalene methyl ester derivative was not (Wainer and Doyle, 1984a, 1984b). The dipole moment of an ester is apparently one-half that of an amide and not strong enough for interaction with the stationary phase.

Various other mechanistic aspects of resolution of diastereoisomers has been reviewed by Gil-Av and Nurok (1974). An impetus to use direct resolution chromatography in recent years has taken its toll with regard to reviews and critical evaluation of diastereoisomeric separation.

The Karger-Halpern-Westley mechanism is still valid for a variety of diastereoisomers. One of the advantages of this mechanism is its ability to predict and correlate the order of elution with configuration, prior to chromatography (Karger et al., 1967; Westley and Halpern, 1968). This, of course, also enables one to choose an appropriate chiral derivatizing agent (CDA).

1.2.1.1.1.1.c ADDITIONAL CHARACTERISTICS OF AN IDEAL DIASTEREOISOMER

In addition to resolvability, the diastereoisomers prepared for chromatographic separation should also satisfy the following requirements: (1) facile and quantitative formation with either conservation or complete inversion of configuration of the enantiomers from which the diastereoisomers are derived, (2) thermal stability for GC or compatibility with the mobile phase for HPLC, (3) absence of racemization during chromatography, and (4) although not crucial, but often desirable for preparative purposes, reconversion of the diastereoisomers, in high yields, to the constituent enantiomers.

1.2.1.1.1.2 AUXILIARY CHIRAL DERIVATIZING AGENTS (CDAs)

The importance of ease of formation and quantitative product yield often determines the choice of an appropriate CDA. Many clinically significant drugs contain an amine (primary or secondary), alcohol, or α-substituted carboxylic acid function. It is therefore not surprising that CDAs often incorporate reactive functional groups such as acid chloride, isocyanate, isothiocyanate or amino. The acid chlorides react with amines to yield amides or with alcohols to give esters. Similarly, isocyanates afford urea derivatives with amines and carbamate (urethane) derivatives with alcohols (vide infra).

The ease of diastereoisomer formation without racemization makes N-trifluoroacetyl-S-prolyl chloride (N-TPC) a CDA of choice for chiro-optical primary and secondary amines and O-alkyl esters of amino acids (Karger et al., 1967; Iwase and Murai, 1974a, 1974b; Manius and Tscherne, 1979; Liu et al., 1982). The volatility of the perfluoroacylated proline in conjunction with the conformational rigidity of the proline ring makes N-TPC a valuable derivatizing agent for gas chromatographic analysis. The native fluorescence property of proline enables N-TPC to be used as a suitable reagent for HPLC with the possibility of improved sensitivity (Sankey et al., 1984).

The stability of N-TPC has caused some concern. Although neither racemization nor asymmetric induction was observed during derivatization (Manius and Tscherne, 1979), racemization during storage (4°C) or synthesis could not be completely ruled out (Gal and Sedman, 1984). Adams and co-workers (1982) attempted to overcome the problem of racemization by utilizing N-trifluoroacetyl prolyl anhydride or N-trifluoroacetylprolyl imidazolide; they were not completely successful as there was partial racemization and, compared to N-TPC, these alternative reagents were sluggish in terms of reactivity.

It has been suggested that storage of N-TPC at -20°C under a nitrogen atmosphere prevents any racemization (Dieterle and Faigle, 1982).

Both isomers of α-methylbenzyl isocyanate (MBI) are commercially available in high optical purity. The problem of racemization is not encountered with these reagents and they are quite stable chemically (Thomson et al., 1982). Several β-blocking (Thomson et al., 1982; Gulaid et al., 1985) and β-lactam (Pirkle et al., 1984c) drugs as well as various secondary alcohols (Periera et al., 1971; Gal et al., 1981; Prelusky et al., 1982) have been separated as diastereoisomeric urea or carbamate derivatives.

In theory, any enantiomer of high optical purity, possessing a reactive functional group, may be used as a CDA. Listed below are several chiral derivatizing agents which have been reported in the recent literature.

1. 2,3,4,6-tetra-O-acetyl-β-D-glucopyranosyl isothiocyanate (GITC) (Nimura et al., 1980; Gal and Murphy, 1984; Grech-Belanger et al., 1985)
2. (R)-α-methylbenzyl isothiocyanate (Gal and Sedman, 1984)
3. (S)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (Sedman and Gal, 1984)
4. R-(-)-O-methylmandelic acid chloride (Hoffman et al., 1983)
5. (-)-camphanic acid chloride (Raud-Christensen and Salvesen, 1984)
6. (-)-menthyl chloroformate (Prelusky et al., 1982; Jeyaraj and Porter, 1984)
7. N-carbobenzyloxy-L-proline (Banfield and Rowland, 1983, 1984)
8. (-)-1-(4-dimethylamino-1-naphthyl)ethylamine (DANE)(Goto et al., 1982)
9. (4R, 5R)-(+)-2-chloro-4,5-dimethyl-1,3,2 dioxaphospholane-2-oxide (Anderson-Shapiro Reagent)(Anderson and Shapiro, 1984)

1.2.1.2.0.0 THE DIRECT METHOD OF CHROMATOGRAPHIC RESOLUTION OF ENANTIOMERS

The direct method of chromatographic enantiomer resolution involves the separation of isomers on a chiral stationary phase (CSP) in which is incorporated an auxiliary resolving agent of high (but not necessarily complete) enantiomeric purity. Chiral recognition is caused by the molecular association between the racemic selectand and the optically active selector in the stationary phase (Schurig, 1984). The chemical nature of the selector determines the type of binding forces that bring about the molecular association required for enantiomeric resolution. In this respect, direct chromatographic resolution may be accomplished by either of two, well established, complimentary techniques: (1) resolution of enantiomers on optically- active amino acid or peptide selectors by means of hydrogen bonding (Gil-Av, 1966; Frank et al., 1978a; Bonner and Blair, 1979; Pirkle et al., 1980, 1981; Konig and Benecke, 1981), and (2) resolution of enantiomers on optically active metal chelates by co-ordination chromatography (complexation chromatography or ligand exchange chromatography (LEC)) (Rogozhin and Davankov, 1971; Schurig, 1977; Moriyasu et al., 1980). The first technique is perhaps more popular because of the commercial availability of several types of chiral (especially HPLC) columns. A third alternative that may soon be available for routine enantiomeric separation is resolution by chiral ion-pairing reagents (Pettersson, 1982, 1983, 1984; Lindner et al., 1984). This technique is presently restricted to HPLC.

1.2.1.2.1.0 MECHANISMS OF RESOLUTION ON CHIRAL STATIONARY PHASES (CSPs)

Separation of enantiomers is achieved by the formation of a transitory diastereoisomeric complex. The development of most CSPs is based upon the original postulate of Dalgliesh (Dalgliesh, 1952). For separation to occur, it was suggested that a minimum of three simultaneous interactions between the CSP (selector) and the solute (selectand) are required and that at least one of these interactions must be stereochemically controlled (as a result of either attractive or repulsive forces). When such an association takes place, a particular conformation is imposed on the solute in which the selector and selectand form parts of a spiral turn, the handedness of which is determined by the configuration of the selector in the stationary phase. The enantiomers thus become conformational diastereoisomers (Feibush and Gil-Av, 1970).

The forces that bring about these three-point interactions may differ from CSP to CSP. The variables and alternative mechanisms will be discussed in more detail in the Sections following.

1.2.1.2.2.1 THE GAS CHROMATOGRAPHIC COLUMNS-CSPs CONTAINING PEPTIDE PHASES

The gas chromatographic columns which contain amino acids as components of the chiral stationary phase may be further categorized into three types:

1. peptide phases (Gil-Av et al., 1966; Feibush and Gil-Av, 1970; Bonner and Blair, 1979)
2. diamide phases (Frank et al., 1978a; Konig et al., 1981)
3. ureide phases (Lochmuller and Souter, 1975)

The development of these phases has been reviewed (Lochmuller and Souter, 1975; Liu and Ku, 1983; Schurig, 1984). The forces responsible for selectand association with the above selector phases are generally of the hydrogen bonding type although dipole-dipole interactions and dispersion forces should also be considered (Stolting, 1976; Abe, 1978).

The Chirasil-Val and RSL-007 columns are the only products presently available commercially. The former seems to be more popular probably because it was available sooner. Chirasil-Val is a co-polymer of dimethylsiloxane and (2-carboxypropyl)siloxane coupled with either L- or D-valine-t-butylamide (Köppenhoeffer and Bayer, 1985a, 1985b). The chiral centres are separated by approximately seven dimethylsiloxane units; hydrogen bonding interactions between neighbouring valine residues are thus avoided. If the separation is decreased, the co-operative effect between the two chiral selectors for the enantiomeric discrimination of the selectand begins to decrease. The monomeric form of diamides have greater resolution properties. Accordingly, the probability of intercalation of solutes between two diamide residues depends upon the distribution function for the chiral groups and the geometry of the polysiloxane chain (Koppenhoefer and Bayer, 1985b). Geometrical and mathematical calculations dictate that the diamide in the Chirasil-Val can exist either in an R-α-pleated helix or a β-pleated helix. The conformation adopted was the one which formed a suitable complex with a given solute via induced fit.

In Figure 2 is depicted the formation of a β-pleated sheet, by interaction with a lactic acid derivative (Frank et al., 1978b). This structure may be further stabilized by Van der Waals forces (Liu and Ku, 1983).

The oxygen-containing enantiomers with no amide function, however, can be completely resolved only if at least two ester carbonyl groups are present (Koppenhoefer and Bayer, 1985a). The separation of such bifunctional oxygen-containing selectands occur in the α-helix of the selector.

The Chirasil-Val column has the disadvantage that its oven temperature range is 70-230°C although it may be operated at 250°C for short periods of time. Racemization of the L-valine component at 230°C (24 hours) is about 3% (Frank et al., 1978c).

The RSL-007 (Chrompac) column, like Chirasil-Val incorporates a diamide phase. L-Valine-S-α-phenylethylamide, as a selector, is covalently linked to cyanoethyl side chains of polysiloxane XE-80 (Konig et al., 1981, 1983) and cannot be operated at temperatures above 165°C.

1.2.1.2.3.0 RECENT TRENDS IN GAS CHROMATOGRAPHIC CHIRAL STATIONARY PHASES

Much of the recent literature deals with the development of diamide CSPs which exhibit improved thermal stability. The incorporation of L-valine-t-butylamide into OV-225 did not improve thermal stability although the resolution properties were comparable to that of Chirasil-Val (Saeed et al., 1979). Similarly, N-lauroyl-L-proline-t-butylamide and higher alkyl diamide phases based on L-valine or L-leucine (Charles and Watabe, 1984) did not offer any advantages over Chirasil-Val. Other recent reports describe N-(1R,3R)-trans-chrysanthemoyl-(R)-1-(α)-naphthylethylamine (Oi et al., 1981), (R)-N-lauroyl-α-(1-naphthyl)ethylamine (Watabe and Gil-Av, 1985), and carbonyl bis(amino acid) ester (Lochmüller and Hinshaw, 1980) CSPs. A review of CSPs has recently been published (Oi, 1984).

1.2.1.2.4.0 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC CSP COLUMNS

In contrast to GC-CSP columns, a variety of chemically different HPLC-CSP columns are commercially available; this represents a significant advantage with respect to the selection of an appropriate phase for the separation of enantiomers.

The first commercially viable HPLC-CSP column was the one introduced by Pirkle in 1981. Pirkle columns are available as Baker Bond Chiral Phase columns in which the selector, (R)-N-3,5-dinitrobenzoylphenylglycine, is ionically or covalently bonded to aminopropyl silica packing (Armstrong et al., 1984; Wainer and Doyle, 1984a, 1984b, 1984c). Chemically related DNBLeu columns, also available from Baker, incorporate an (S)-N-3,5-dinitro-benzoylleucine phase. These CSPs utilize a combination of pi acidity, hydrogen bonded receptor sites and hydrogen bond donor sites to fulfil the three point interaction requirement. Dipole-dipole intercalative processes play an important role in determining enantioselectivity. Recent developments include conformationally rigid urea- and hydantoin-based CSPs (Pirkle, 1985d). As discussed earlier, conformational rigidity is expected to improve isomer separation (Pirkle et al., 1981, 1984a, 1984b, 1984c, 1984d; Pirkle and Hyun, 1985a, 1985b, 1985c).

Oi et al. (1983a, 1983b) and Oi and Kitahara (1983) have developed a series of chiral stationary phases particularly designed for 3,5-dinitrophenyl derivatives of alcohols and amino acids. These phases contain S-(1)-α-(naphthyl)ethylamine or 2-(4-chlorophenyl)isovaleric acid bonded to aminopropyl silanized silica. This research group is also responsible for (1R, 3R)-trans-chrysanthemic acid- and amide-derived stationary phases (Oi et al., 1983c). These phases have exhibited good resolving properties towards amines, esterified amino acids, alcohols and carboxylic acid esters.

1.2.1.2.5.0 NOVEL CSPs IN HPLC

The possibility that proteins might adsorb enantiomers to different degrees led to the development of protein-based columns. Hermansson (1983) reported the use of α_1-acidglycoprotein (orosomucoid) and Allenmark and Bomgren (1982) and Allenmark et al., (1982) demonstrated the use of agarose albumin as chiral stationary phases for enantiomer separation via affinity chromatography. These CSPs depend on hydrophobic forces for selector-selectand interactions and therefore pH, mobile phase composition (Hermansson, 1983; Allenmark and Bomgren, 1982; Allenmark et al., 1982, 1983, 1984b) and protein concentration (Allenmark et al., 1984a) influence the stability of three point-interaction bonds.

Although several cellulose-derived CSPs have been developed (Luttringhaus et al., 1967; Yausa et al., 1974; Hess et al., 1978; Hakl et al., 1979), only acetylcelluloses presently have practical and commercial viability (Lindner an Manschrek, 1980; Blaschke, 1983). Triacetylcellulose HPLC phases, as well as corresponding cross-linked capillary GC phases (Klemisch and VanHodenberg, 1985) depend upon crystallinity for chiral discrimination (Hesse and Hegle, 1973, 1976a, 1976b; Okamato et al., 1984a; Francotte et al., 1985a). In HPLC, the chromatographic separations are influenced by the linear velocity and composition of the mobile phase (Mannschreck et al., 1983).

The triacetylcellulose phases have been used for the stereoisomeric separation of geometrical isomers (Prelog and Weidlund, 1984; Mannschreck et al., 1983; Shlogl and Widhalm, 1984), compounds of metabolic interest such as ephedrine, mandelic acid esters as well as such drugs as piprozolin, etozolin, and oxindazac (Klemisch and VanHodenberg, 1985; Francotte et al., 1985b).

1.2.1.2.6.0 DIRECT RESOLUTION BY COMPLEXATION IN GAS CHROMATOGRAPHY

Resolution by complexation relies on fast and reversible interaction (complexation or co-ordination) between the solute and transition metal additives. Selectivity is dependent upon the proper selection of an appropriate metal chelate. (Schurig and Weber, 1984).

Schurig (1977) accomplished the first gas chromatographic resolution of enantiomers on a capillary column loaded with a squalene solution of optically active dicarbonylrhodium (I)-3-trifluoroacetyl-(1R)-camphorate.

Recent developments include the use of thermally stable bis-3-(perfluoroacyl)-1R-camphorates of manganese (II), cobalt (II) and nickel (II) for the resolution of various compounds of natural and synthetic origin (Schurig and Burkle, 1978; Schurig et al., 1979; Koppenhoefer et al., 1980; Schurig and Weber, 1981; Weber and Schurig, 1984). Introduction of fused silica columns with active metal chelates will avoid any interaction between metallic tubing material and the chelate (Schurig and Weber, 1984a) and thereby permit charge-transfer interactions only between the selector and the selectand (Oi et al., 1981a).

The mechanistic aspects of chromatographic separation on optically active metal complexes has been recently reviewed by Yoneda (1985).

1.2.1.2.7.0 DIRECT RESOLUTION BY LIGAND EXCHANGE IN HPLC

There are basically two approaches to the chiral resolution of enantiomers by ligand-exchange HPLC. The chiral selector is either present as a chemically bonded HPLC phase or as an additive in the mobile phase. The bonded phases are utilized for both analytical and preparative purposes (Feibush et al., 1983). The presence of a chiral selector in the mobile phase frequently results in high enantioselectivity and efficiency. In addition the resolving agent is easily removed thereby allowing the column to be used for achiral purposes (Lam, 1984).

Ligand exchange chromatography (LEC) columns for HPLC (Bou'e et al., 1981) contain an optically active ligand packing material frequently composed of copper-complexed L-amino acids either chemically bonded (Gubitz et al., 1981a, 1981b) or simply coated on silica beads. Cyclic amino acids, as selectors in LEC, have also been successfully employed (Davankov et al., 1979; Gubitz et al., 1982; Busker and Martens, 1984).

The process of chiral recognition involves the formation of kinetically labile, bidentate complexes on the stationary phase (Rogozhin and Davankov, 1971). Differences in the stabilities of the complexes lead to separation of the enantiomers (Gubitz et al., 1981a, 1981b). The pH of the eluent often determines the ease of formation of the complexes (Davankov and Kurganov, 1983) while ionic content is more important for peak shape and symmetry (Gubitz et al., 1981a; Davankov et al., 1983). The metal ion (usually copper, but also mercury) may compete for the protons from the selectand (Lindner et al., 1979; LePage et al., 1979) and therefore influence the capacity factor (Lefebvre et al., 1978; Sugden et al., 1980, 1981; Charmot et al., 1985b). It has been demonstrated that a metal to chelate ratio of 1:1 provides optimal resolution (Feibush et al., 1983). Introduction of compounds, such as ammonia, which are capable of forming monodentate complexes with metallic ions can also significantly increase the HETP values (Charmot, 1985a, 1985b).

An alternative technique is to add the optically active ligand directly to the mobile phase (in a metal to amino acid molar ratio of 1:2) for either an ion-exchange (Hare and Gil-Av, 1979) or reversed phase HPLC (Wernicke, 1985).

The chiral separation takes place through mixed chelate complexation (Lam, 1984), which usually takes the form of a ternary complex (Yamauchi et al., 1979) in the mobile phase. The formation of a ternary complex has been explained by two theories, namely: (1) the theory of outer sphere complexation (Cook et al., 1978; Chow and Grushka, 1979; Lindner et al., 1979) and, (2) the ligand field exchange stabilization energy theory (Wernicke et al., 1985).

Resolution is affected by pH, hydrophobicity of the selectand and complex stability (Gilon et al., 1981); it may be improved via metal complex concentration gradients (Lam, 1984).

Various amino acids, such as L-hydroxyproline (Busker and Martens, 1984), L-phenylalanine and D-phenylglycine (Nimura et al., 1981a, 1981b , 1982; Forsman, 1984) and the disaccharide aspartame (Gilon et al., 1979) have been used as selectors in mobile phase LEC. Other developments include post-column derivatization techniques (Weinstein et al., 1982) and micro-column technology using L-histidine complexes (Takeuchi et al., 1985).

1.2.1.2.8.0 DIRECT RESOLUTION BY INCLUSION COMPLEXATION IN HPLC

Cyclodextrins (CXD) are chiral, toroidal-shaped molecules, containing 12 glucose units bonded through α-(1,4)-linkages. The smaller homologs, i.e. α-CXD (6 glucose units), β-CXD (7 glucose units) and γ-CXD (8 glucose units) are commercially available (Armstrong et al., 1984a). Each glucose unit is arranged so that all the hydroxyl groups are on the outer edges of the molecule (Armstrong et al., 1984b, 1984c, 1984d). Chiral recognition takes place through inclusion complex formation with the selectand, resulting in a tight fit within the hydrophobic cavity of cyclodextrin (Debowski et al, 1983; Daffe and Fastrez, 1983; Armstrong et al., 1985a, 1985b, 1985c; Hinze, et al., 1985). CXDs can also be used as chiral mobile phase components (Debowski et al., 1982, 1983) or as achiral mobile phase modifiers (Debowski et al., 1985).

Apart from chiral resolution, the cyclodextrin-bonded phases have also been used for the separation of such structurally related compounds as benzo(e)pyrene and benzo(a)pyrene, fluorene, carbazole (Armstrong et al., 1985b), ferrocene, ruthecene and osmocene analogs (Armstrong et al., 1985c) as well as for drug discrimination (Han et al., 1984).

1.2.1.3.0.0 CHROMATOGRAPHIC ORDER OF ELUTION AND STEROCHEMISTRY

Several investigators have attempted to correlate configuration with the order of elution of enantiomers and diastereoisomers on chiral and achiral chromatography phases, respectively. It has been suggested that for closely related compounds the relationship between the order of elution and configuration is generally consistent (Gil-Av and Nurok, 1974; Schurig, 1984), although exceptions have been reported (Konig et al., 1984b).

On the basis of data obtained from a study of the relationship between the order of emergence of amino acids and 2-aminoalkanes on an optically active carbonyl bis-(N-L-valine isopropyl ester) stationary phase, Feibush and co-workers (1972) formulated the following rule: the enantiomer emerging first has the substituents at the asymmetric carbon arranged counterclockwise in descending order of size, when the molecule is viewed in the direction from the carbon to the nitrogen atom (i.e. the configuration is R). This observation was further substantiated by Rubinstein (1973) who determined that the R-enantiomers of 2-aminoalkanes had shorter retention times than S-enantiomers. Binet and co-workers (1984) established a correlation between the order of emergence and the absolute configuration of secondary alcohols using (+)-dodecyl-(2R,3R)-tartrate as the stationary phase. The first-eluting enantiomer was the alcohol in which the three substituents were arranged in a clockwise direction according to decreasing size when the molecule was observed in the direction from the asymmetric carbon to the oxygen atom. However, since [illegible] above observation was based only upon the bulkiness of th[illegible]bstituents, it may not bear any correlation with the configuration.

Helmchen and co-workers (1979a, 1979b) determined that in the absence of specific solvating effects or solute intramolecular interactions, solute molecules tend to align with the solvent molecules. In addition, solute molecules favor conformations that have the lowest internal potential energy. Thus, of a pair of diastereoisomers, the one that achieves an alignment approximating the preferred rotational conformation will be retained longer by the solvent (Sonnet, 1984). Sonnet and Heath (1982) reported similar observations upon studying the resolution behaviour of diastereoisomeric amides and carbamates. With carbamates, the carbonyl -containing functional group serves to create a plane between the asymmetric centers which have alkyl(aryl) groups extending to either side of that plane. Those diastereoisomers which feature the largest groups on the same side of the plane ('cisoid' diastereoisomers) (Fig. 3) are able to more easily approach the stationary phase from that planar face which has the smaller alkyl or aryl residues exposed.

The hydrophobicity of the substituents may result in some degree of repulsion and in extreme cases the usual elution order is reversed. The situation for amides is analogous to that of carbamates; the asymmetric centres are separated by one less atom and 'cisoid' diastereoisomers often have the R,R-configuration. This 'cisoid, transoid' concept agrees with the HPLC elution orders observed by Pirkle and Hauske (1977). The elution orders were exactly reversed in gas chromatography with the 'transoid' diastereoisomers being retained longer (Sonnet and Heath, 1982). In the same study, the trifluoromethyl-substituted diastereoisomeric carbamates eluted much more rapidly than their methyl counterparts. The elution orders were reversed, with the 'cisoid' being retained for longer periods. Similar observations were made on HPLC (Pirkle and Hauske, 1977) and were ascribed to the considerable hydrophobicity of the CF_3 group. Even though the group is small, the repulsion from the hydroxyl groups of the silicone in the stationary phase causes a decrease in the capacity factor and a reversal in the order of elution.

1.2.2.0.0.0 CHROMATOGRAPHIC RESOLUTION OF SYNTHETIC CHIRO-OPTICAL XENOBIOTICS

In the preceeding Sections a conceptual overview of various aspects of chromatographic enantiomer resolution was presented. The suitability of chromatography for sensitive and precise determination of enantiomers has resulted in its widespread application to the pharmaceutical and medical fields. An overview of the chromatographic techniques utilized for the resolution of diverse synthetic xenobiotics is now presented. For the purposes of discussion, the following topics are emphasized since they are particularly pertinent to the research presented in this thesis:

1. amphetamines and related amines
2. arylalkanoic non-steroidal antiinflammatory drugs (NSAIDs)

The chromatographic separation of various medically important compounds is summarized in Tables 1-7. No attempt has been made to include anti-infectious agents or products of natural origin.

1.2.2.1.0.0 AMPHETAMINE-LIKE CNS STIMULANTS

(+/-)-α-Methylbenzeneethanamine (amphetamine) is an interesting drug with respect to the simplicity of its structure but diversity of biological effects. As a result, this drug has been a ready target for extensive molecular modifications and metabolic studies in order to accentuate some biological effects but abolish others.

The stereochemical aspects of (+/-)-amphetamine metabolism were investigated as early as the 1940's (Beyer et al., 1940; Harris et al., 1947) but it was not until 1967 that a sensitive and stereospecific GC analytical method was developed (Gunne, 1967). In that study, the proportion of the optical isomers of amphetamine present in human urine was determined by GC of the N-TPC-derived diastereoisomers. Forensic interest in the drug (State v/s McNeal, 1980) encouraged the further development of stereospecific analytical methods for amphetamine and its analogs. Following a comparative study (Liu et al., 1982), the use of direct or indirect capillary GC/MS was advocated for forensic studies. It was determined that the combination of an achiral column and a chiral derivatizing agent (N-TPC in this case) was adequate for analyzing mixtures of S-(+)- and R-(-)-amphetamine.

Pirkle and Simmons (1983) investigated the use of chiral 4- and/or 5-aryl substituted 2-oxazolidonecarbamyl chlorides for HPLC resolution of racemic primary amines. The most favorable separation occurred when the 2-oxazolidone aryl substituent was at the 4- rather than 5-position. In addition, α-naphthyl substituents were more effective than phenyl groups. However, it was noteworthy that two phenyl groups in a cis-4,5-relationship generated a larger α-value than a single naphthyl substituent.

Gal and colleagues (Miller et al., 1984) investigated the HPLC resolution of racemic amphetamine and phenylethylamines after derivatization with the following chiral reagents: R-(+)-1-phenylethyl isocyanate (PEIC), (-)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MPTA-Cl), 2,3,4,6-tetra-O-acetyl-β- D-glucopyranosyl isothiocyanate (GITC) and 2,3,4,-tri-O-acetyl-α-D-arabinopyranosyl isothiocyanate (AITC). Reactions were conducted under mild conditions (25-70°C) and were complete within one hour. In general, HPLC resolution of the diasteroisomeric products of GITC, AITC and MPTA-Cl was more complete than with PEIC. According to the authors, these methods were adequate for biological analyses; less than 100 ng of a derivative (on column) could be detected by its UV absorption at 254 nm.

In an earlier study, Gal (1977) examined the GC separation of (+/-)-amphetamine and eight closely related compounds, using the chiral acylating agent MPTA-Cl. Under the GC conditions used, all the amines were resolved with the exception of the hydrazine analogs of amphetamine and tranylcypromine. A definite relationship was observed between the absolute configuration and order of elution of the diastereoisomers; the R-isomer always eluted earlier than the S-isomer, a situation analogous to N-TPC derivatives of amphetamines (Liu et al., 1982). It is interesting to note that N-drimanoylamphetamine diastereoisomers (derived from (R)-drimanoyl chloride) exhibited a reversal in the order of elution compared to prolyl amide or chrysanthemic amide derivatives (Brooks et al., 1973).

Barksdale and Clark (1985) have recently described the use of 4-nitrophenylsulfonyl-(S)-prolyl chloride (NPSP-Cl) as a CDA for reversed phase HPLC analysis of (+/-)-amphetamine. This reagent was prepared in a two-step reaction from L-proline and 4-nitrophenylsulfonyl chloride (Clark and Barksdale, 1984).

All of the HPLC methods described above utilized UV absorption for detection. Mutschler and co-workers (Weber et al., 1984) developed an HPLC method using S-(+)- or R-(-)-benoxaprofen (acid chloride) as sensitive, stable chiral fluorescence markers. This method was suitable for the simultaneous stereospecific determination of racemic amphetamine, methylamphetamine and tranylcypromine. The authors observed that the intrinsic fluorescence properties of benoxaprofen enhanced sensitivity and that the configurational rigidity of benoxaprofen resulted in 'chiral stability' and therefore better separation. The enantiomers of benoxaprofen could be stored at room temperature for up to twelve months without racemization. The HPLC (Wainer et al., 1984) and HPLC/MS (Crowther et al., 1984) separation of (+/-)-N-naphthoylamphetamine (and derivatives) on Pirkle columns has also been reported.

Recently HPLC separation of (+/-)-threo-methylphenidate was reported (Lim et al., 1985). The method used d-10-camphorsulphonic acid as the chiral counterion additive, and UV detection. This technique of ion-pair chromatography is an established method for routine resolution and optical separations by HPLC. The optical separations are dependent upon the formation of diasteroisomeric ion-pairs, with the chiral counter ion in the mobile phase as a chiral additive. The diastereoisomeric ion-pairs are separated on conventional non-chiral solid phases using an organic mobile phase of low polarity in order to promote a high degree of ion-pair formation (Pettersen, 1984). Diastereoisomers of methylphenidate (i.e. R,R- and R,S-isomers) are themselves resolvable under achiral conditions (Padmanabhan, 1980).

(+/-)-Fenfluramine (N-ethyl-m-trifluoromethyl-amphetamine) is an anorectic agent which differs from amphetamine in that it causes depression rather than stimulation of the CNS (Midha, et al., 1983). The major route of elimination is by N-deethylation (Beckett and Brooks, 1967) to norfenfluramine. For stereospecific analytical purposes, it has been reported that reactions with optically pure N-TPC or pentafluoropropionyl prolyl chloride yield fenfluramine and norfenfluramine diastereoisomers which are highly sensitive to GC electron-capture detection (Caccia and Jori, 1977). In addition, polar GC phases (e.g. OV 225) were much more effective in achieving resolution than slightly polar (OV 17) or non-polar phases. This method was subsequently used to investigate the disposition and species differences in the stereoselective kinetics and biotransformation of fenfluramine isomers (Jori et al., 1978; Caccia et al., 1981, 1982).

1.2.2.2.0.0 CHIRO-OPTICAL NON-STEROIDAL ANTIINFLAMMATORY DRUGS: THE ARYLALKANOIC ACIDS

The non-steroidal antiinflammatory arylalkanoic acids are widely used therapeutic agents. A recent review (Verbeeck et al., 1983) as well as numerous reports describing analytical procedures (Mori et al., 1983, 1985;

Litowitz et al., 1984; Heikennen, 1984; Albert et al., 1984a, 1984b) and kinetic investigations (Greenbelt et al., 1984; Au et al., 1984; Houghton, 1984; Jamali et al., 1984, 1985) add credence to the clinical importance of these drugs. It is interesting to note that in none of the above studies was the racemic nature of these agents mentioned. Metabolic stereospecific inversion from the R- to S-configuration (Israili, 1977; Hutt and Caldwell, 1983) calls for sensitive chromatographic methods capable of determining the relative proportions of R- and S-enantiomers present in metabolic studies. Furthermore in the event that in vivo optical inversion does occur, the pharmacokinetic, therapeutic, pharmacological and toxicological implications of such a process must be considered.

Among the arylpropionic acid group of drugs, ibuprofen was the first compound to be investigated stereochemically. Vangiessen and Kaiser (1975) developed a procedure for the quantification of S-(-)-α-methylbenzylamide derivatives of ibuprofen by GC-flame ionization detection (FID). The method was capable of quantifying 1 ug of each enantiomer per mL of the biological sample (plasma or urine). The procedure was also applicable to human drug absorption studies (Kaiser et al., 1976). Structures of the diastereoisomeric methylbenzylamides were confirmed by

GC/MS analysis. Other arylpropionic acids which have been similarly derivatized for GC or HPLC analysis include 2-phenylpropionic acid, 2-(2-naphthyl)propionic acid, naproxen, 2-(4-biphenyl)propionic acid, flurbiprofen, cicloprofen, carprofen, suprofen, benoxaprofen, and fenoprofen (Kemerer et al., 1979; Bopp et al., 1979; Stottenberg et al., 1981; Maitre et al., 1984; Rubin et al., 1985).

It is interesting to note that methylbenzylamide derivatives of certain arylpropionic acids have been chromatographically resolved by both GC and HPLC. These NSAIDs include ibuprofen (Maitre et al., 1984 (HPLC); Kaiser et al., 1976 (GC)), benoxaprofen (McKay et al., 1979 and Simmonds et al., 1980 (HPLC); Bopp et al., 1979 (GC)), and indoprofen (Tamassia et al., 1984 (HPLC); Tossolini et al., 1972 (GC)).

In some cases the low volatility and thermal instability of NSAID methylbenzylamide derivatives make resolution by GC difficult. In such situations, HPLC is an attractive alternative analytical procedure. The diastereoisomeric derivatives are prepared by condensation of the NSAID with 1,1'-carbonyldiimidazole (CDI) followed by reaction with the chiral amine. Excess CDI is eliminated by addition of acetic acid. Reaction of the arylalkanoic acid with the coupling reagent CDI, is rapid, whereas the subsequent reaction with the chiral amine is generally slower and requires about 30 min for completion (Vangiessen and Kaiser, 1975). Testa and co-workers suggested that prior to their study (Maitre et al., 1984) the carbonyldiimidazole was being used in sub-optimal proportions. Increasing the amount of CDI from 6.5 mg to 52 mg per reaction mixture (containing between 0.05 to 0.5 ul of the arylalkanoic acid) resulted in much better yields of the derivatives and hence enhanced sensitivity. These workers utilized normal phase HPLC with UV detection.

Condensing agents other than CDI have been used to facilitate amide formation. In order to demonstrate the in vivo optical inversion of (R)-clidanac in guinea pigs, Tamura and co-workers (1981) developed a thin layer chromatographic (TLC) method to quantify the individual enantiomers as their diastereoisomeric R-(+)-α-methylbenzylamides. In this study the amides were formed via condensation with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide. This reaction, however, exhibited an enantiomeric bias since amide formation with R-(-)-clidanac was slightly favoured. The method gave an amide yield of only 60%, primarily as a result of by-product formation rather than incomplete reaction. 1-Ethyl-3-(3-dimethyl- aminopropyl)carbodiimide has also been used for condensation of naproxen with a fluorophore chiral inductant, (-)-1-(4-dimethylamino-1-naphthyl)ethylamine (DANE) (Goto et al., 1980, 1982). This derivative was highly sensitive (100 pg naproxen in serum) to HPLC fluorescence detection (excitation wavelength 320 nm; emission wavelength 410 nm). The yield of the final derivative was further improved by the addition of 1-hydroxybenzotriazole, a racemization suppressant. The method was also applicable to racemic ibuprofen and indoprofen. In another study, indoprofen was coupled with L-leucinamide following ethyl chloroformate-mediated anhydride formation (Bjorkman, 1985). The reaction was complete within 3 min with no detectable racemization. Resolution was achieved on a reverse phase HPLC system; switching from UV to fluorescence detection did not improve the sensitivity.

In an earlier study, L-leucine was used as a CDA for the radio-iodometric analysis of labelled cicloprofen (Lan et al., 1976). The radio-iodometric method was developed as an alternative to optical rotation and circular dichroism measurements (Kripalani et al., 1976).

As an alternative to the use of coupling reagents, the arylalkanoic acids may be reacted with thionyl chloride (to give the respective acid chlorides) followed by an optically pure amine. The methylbenzylamide derivatives of benoxaprofen (McKay et al., 1979; Simmonds et al., 1980) and indoprofen (Tossolini et al., 1972; Tamassia et al., 1984) were prepared by this route for the purposes of studying their stereoselective disposition in mammals. However, the use of thionyl chloride has limitations as described by Kaiser et al. (1976). Thionyl chloride was found to dehydrate the hydroxy metabolite, 2,4'-(2-hydroxy-2-methylpropyl)phenylpropionic acid, of ibuprofen. The reagent also chlorinated this metabolite and the resulting product then dehydrochlorinated.

Compared to those involving optically active methylbenzylamine, there are relatively few reports describing other CDAs for the separation of arylpropionic acid enantiomers. Lee and co-workers (1984) resolved the S-(+)-2-octyl esters of R- and S-ibuprofen on an HPLC system using a normal phase column with UV detection. The method was subsequently used to study the stereoselective disposition of ibuprofen in humans (Lee et al., 1985). The arylacetic acid derivative, etodolac, has been resolved via liquid chromatographic separation of the diastereoisomeric (-)-borneol esters (Demerson et al., 1983). The enantiomers of 2-[3-(2-chlorophenoxyphenyl)]propionic acid have been determined as amide derivatives of optically pure 2-aminobutane; in this study the intermediate acid chloride was prepared with oxalyl chloride (Tamegi, 1979b).

Direct liquid chromatographic resolution of arylpropionic acids has also been described (Wainer and Doyle, 1984a, 1984b; Crowther et al., 1984). After examining various achiral amide derivatives, it was determined that secondary amines (e.g. N-methylbenzylamine and N-methyl-1-naphthylamine) were the most suitable derivatizing reagents (Wainer and Doyle, 1984b). The amide derivatives were then resolved on a Pirkle column. In the same study, the authors were unable to resolve the NSAIDs as their aromatic ester derivatives.

Crowther et al. (1984) compared the relative performances of two Pirkle (J.T. Baker- and Regis-packed) columns. Although adequate separation of benzylamide derivatives of ibuprofen was accomplished in each case, the Baker-packed column provided adequate separation in about half the time. The study subsequently led to an investigation of the stereochemical composition of ibuprofen in horses using an LC/MS system.

It is evident that the enantiomers of NSAID arylalkanoic acids are best separated as amide derivatives which are, in turn, prepared by means of a condensing or coupling agent. The coupling agents have an advantage over acid chloride-forming reagents in that they are very mild and do not cause side reactions with labile metabolites. Although a detailed mechanistic discussion on the use of various coupling agents is outside the scope of this thesis, the interested reader is referred to the following papers: Staab (1956), Anderson (1958), Winridge and Jorgensen (1971), Bosshard (1973), and Takeda et al. (1985).

1.3.0.0.0.0 STEREOSELECTIVE DISPOSITION OF CHIRAL XENOBIOTICS IN MICROBIAL AND MAMMALIAN SYSTEMS

In previous sections various aspects related to the chromatographic separation of stereoisomers have been described. The remainder of the introduction will focus on selected routes of microbial or mammalian metabolism which illustrate the importance of drug stereochemistry in metabolic pathways. For the purposes of this thesis, the following metabolic processes were selected:

1. microbial p-hydroxylation of β-adrenoceptor drugs
2. microbial S-oxidation of β-lactam derivatives
3. mammalian N-dealkylation of amphetamine analogs
4. mammalian chiral inversion and disposition of 2-arylalkanoic acids

1.3.1.0.0.0 MICROBIAL MODELS OF MAMMALIAN METABOLISM

Microbial transformation is a term used to describe the metabolism of chemicals by microorganisms. Initially studies were performed in an effort to discover new substances. This led to the realization that various transformation reactions were common to mammals and microorganisms (Reighard and Knapp, 1986).

The concept of using microbial systems to study mammalian metabolism was first introduced in 1974 (Smith and Rosazza, 1974). The method has several advantages. In addition to the economical and ethical benefits, microbial transformations are frequently regioselective/specific and stereoselective/specific (Kieslich, 1980; Clark et al., 1985).

Microorganisms may serve as effective models for mammalian metabolism since the cytochrome P-450 monooxygenase enzymes are similar to those found in mammalian liver (Ferris et al., 1976; Cerniglia et al., 1978; Rosazza and Smith, 1979). As a multitude of cytochrome P-450-mediated biotransformations is possible, the discussion in the sections following will be restricted to oxidative pathways and, more specifically, aromatic hydroxylation and sulfur oxidation.

1.3.1.1.0.0 MICROBIAL p-HYDROXYLATION OF β-ADRENOCEPTOR DRUGS

Aromatic hydroxylation is a common metabolic route in humans. The steroids (Matsubara et al., 1975), amphetamines (Coutts et al., 1976, 1977, 1978, 1984; Cho, 1975), niflumic acid (Lan et al., 1975), mepivacaine (Meffin et al., 1973) and other antiarrhythmic agents (Beckett and Chidomere, 1977; Farid et al., 1983), phenytoin (Chow and Fischer, 1982), tiaprofenic acid (Jamali et al., 1984, 1985; Sorkin et al., 1985), propranolol (*vide infra*), chlorprothixene (Breyer-Pfatt et al., 1985), debrisoquine (Kahn et al., 1985), and hexabarbital (Miyano, 1980) are just a few ~~of the~~ drugs which undergo this metabolic process.

Pasutto and co-workers reported the exclusive formation of the p-hydroxy metabolites of propranolol and toliprolol from *Cunninghamella echinulata*-mediated reactions (Nazarali et al., 1982). Since it was of interest to further illustrate the microbial model of mammalian metabolism as well as microbial metabolic (Fig. 4) stereoselectivity, it was felt that in view of the structural similarities between propranolol and pre-prenalterol, *C. echinulata* would similarly metabolize pre-prenalterol to afford prenalterol.

Propranolol is a non-selective, β-receptor antagonist which is metabolized extensively by man, dog and rat (Walle et al., 1978, 1985; Bourne et al., 1981; Barger et al., 1983; Bai and Walle, 1984). Several metabolic processes are observed, including N-dealkylation of the propanolamine side chain as well as O-alkylation and aromatic hydroxylation (Bond, 1967; Walle and Gaffney, 1972; Tindell et al., 1972; Walle et al., 1972; Pritchard, 1979). The principal product of aromatic hydroxylation is 4-hydroxypropranolol which is believed to contribute to the pharmacological and therapeutic effects of propranolol (Zacest and Koch-Weser, 1972; Mehta et al., 1983). Other ring hydroxylated metabolites of propranolol have also been isolated after either in vitro (Walle et al., 1974; Tindell et al.,1978) or in vivo experiments (Walle et al., 1978, 1982).

Microbial monooxygenase systems are believed to be similar to those in mammals and it would be appropriate to assume that microorganism-mediated aromatic hydroxylations proceed through an NIH shift. Although the literature dealing with the NIH shift mechanism in mammals is well referenced (Daly and Jerina, 1969; Jerina et al., 1968; Daly et al., 1968; Jerina et al., 1971; Kaubisch et al., 1972; Bruise et al., 1973; Tomaszewski, 1975; Nelson and Powell, 1979; Walle et al., 1983a, 1984) there are relatively few reports of this mechanism in microorganisms (Smith and Rosazza, 1975).

Prenalterol is a cardioselective β-agonist having a more pronounced effect on myocardial contractility than on heart rate (Kendell et al., 1982). The pharmacological effects are associated with the levo-isomer (Kendall et al., 1982). The pharmacokinetic properties include rapid peak plasma levels, elimination half-life of 2 hours (Ronn et al., 1979, 1980), and excretion of approximately 80% of the drug urine as the sulfate ester (Hoffman et al., 1982).

Prenalterol has been assayed in biological samples after perfluoroacylation using GC-electron capture detection (Degen and Ervik, 1981) and GC/MS (Ervik et al., 1982). Recently, the determination of prenalterol in plasma and urine by HPLC-electrochemical detection has also been performed (Lagerstorm and Carlebom, 1984).

The chemical synthesis of prenalterol has been described (Crowther et al., 1969), but the utilization of microorganisms in the synthetic scheme has not been reported.

1.3.1.2.0.0 **MICROBIAL S-OXIDATION OF β-LACTAM DERIVATIVES**

In mammals, sulfides are metabolized to sulfoxides and sulfones (Beckett et al., 1975; Breyer-Pfaff et al., 1978).

Frequently metabolism is accompanied by a substrate-metabolite interaction (Shavit et al., 1980) or an intermolecular rearrangement associated with C-S bond cleavage (Hanzlek and Cashman, 1983). As suggested by Smith and Rosazza (1975) there are few systematic studies explaining the process of metabolic sulfoxidation. It is very likely that enzyme systems responsible for catalyzing sulfur oxidations (Testa and Jenner, 1978) such as the oxidation of dithiothreitol (Poulsen et al, 1975) are also involved in N-oxidation. The N-oxidation inhibitory activity of dithiothreitol and cysteamine (Beckett, 1971; Zeigler and Mitchell, 1972) and conversely sulfoxidation inhibition by methimazole and phenylthiourea (Hanzlik and Cashman, 1983) indicates the presence of a common enzymatic system.

In general, microbiological sulfur oxidation proceeds in a step-wise manner, through the sulfoxide to the sulfone (Fig. 5). The microorganism-mediated oxidative pathway can be interrupted at the sulfoxide stage using acetone powder (Smith and Rosazza, 1975). Frequently sulfoxidation is regio- and stereoselective (Auret et al., 1968a, 1968b, 1974). The degree of stereoselectivity depends upon the strain of microorganism, as well as such substrate-related factors as stearic hindrance and size. For example, a high degree of optical enrichment is observed during the sulfoxidation of bulky sulfides (Auret et al., 1968b).

Microbial oxidation of sulfur in the cephem nucleus of the cephalosporins was reported in 1980 (Torrii et al., 1980). The Coriolus hirsutus-mediated oxidation of 7-phenylacetamido-3-deacetoxy cephalosporanic acid (7-PhADCA), 7-phenoxyacetamido-3-deacetoxy cephalosporanic acid (7-PhOADCA) and cephalexin yielded sulfoxides with varying degrees of optical enrichment.

The cephalosporin (R or S) sulfoxides have received recent attention due to their reportedly high intrinsic antimicrobial activity compared to the parent drugs(Gorman & Ryan 1972). In addition, regio- and stereospecific chemical syntheses of S-oxides may be difficult and consequently uneconomical (Torrii et al., 1980). Directed microbial synthesis appears to represent a viable alternative.

1.3.2.0.0.0 MAMMALIAN N-DEALKYLATION OF AMPHETAMINE ANALOGS

Fenfluramine, available commercially as a racemate, is an effective anorectic agent in laboratory animals (LeDourec et al., 1966) and humans (Munro et al., 1966). The drug is primarily metabolized by N-deethylation to norfenfluramine (Beckett and Brookes, 1967; Bruce and Maynard, 1968) which also has intrinsic anorectic properties (Beregi et al., 1970; Braekkaup et al., 1975). Species of animal (Campbell et al., 1971), route of administration (Beckett and Salmon, 1972), and stereochemistry (Morgan et al., 1970; Beckett and Brookes, 1970) are known to influence the N-deethylation of fenfluramine. The S-(+)- and R-(-)-isomers of fenfluramine and norfenfluramine, respectively, have different biochemical and pharmacological properties (Beregi et al., 1970; Crunelli et al., 1980; Maizi et al., 1981) and are in fact metabolized at different rates in laboratory animals (Jori et al., 1978; Garattini et al., 1979; Caccia et al., 1981). For a better understanding of this mechanism, it could be more appropriate to review the process of N-deethylation on amphetamines.

All of the N-substituted amphetamines, phentermines and ephedrines undergo N-dealkylation. Quantitatively, this is an important route of metabolism, although it is apparently less extensive in man than other animal species (Caldwell, 1976). N-Dealkylation is an oxidative process catalyzed by the microsomal fraction of the liver. The products are a primary amine and an aldehyde derived from the alkyl group which has been removed (Axelrod, 1955; Dring et al., 1979). The metabolic removal of N-alkyl substituents has been studied extensively. Two basic mechanisms have been proposed; the first proceeds through the formation of a carbinolamine derivative (Fig. 6) (Brodie et al., 1958; McMahon, 1966) while the other involves N-oxidation to an hydroxylamine, which then rearranges to give the carbinolamine (Fish et al., 1955, 1956; Zeigler and Petit, 1966; Machinist et al., 1966; Hendersen et al., 1974). It has been suggested that the first mechanism is most likely although the second may be preferred in certain situations (Hucker, 1973). Alternatively, as in the case of imipramine, both pathways may be simultaneously operative (Bickel, 1969, 1971). It should be mentioned that α-C-oxidation and N-oxidation proceed via different metabolic pathways and involve different enzyme systems (Beckett, 1971).

Of the factors which influence the rate and extent of N-dealkylation, the most important are the nature of the N-alkyl substituent, the stereochemistry of the amphetamine analog and, of course, the animal species. The rate of N-dealkylation in man increases with the size of the N-alkyl group (methyl, ethyl, isopropyl series). The N-n-propyl analog is dealkylated at a slower rate than the iso-analog (Vree et al., 1971a). It has also been demonstrated that the introduction of a m-CF_3 group in ethylamphetamine (i.e. fenfluramine) results in a slight increase in the extent of N-dealkylation. On the other hand, the presence of an additional methyl group on the carbon adjacent to the amine function (phentermine analogs) has a depressing effect on metabolism. In rats it has been shown that N-dealkylation occurs at a moderate rate and side chain oxidation (e.g. β-oxidation) is very limited (Caldwell, 1976). A substituted N-alkyl group may either favor or hinder N-dealkylation. The proportion of amphetamine found in rat brains was higher with N-2-cyanoethyl- (fenproporex) and N-3-chloropropyl(mefenorex) analogs compared to that found after intraperitoneal administration of _n_-propylamphetamine (Nazarali, et al., 1983).

With respect to metabolic stereoselectivity in man, N-dealkylation generally favors S-(+)-N-alkylamphetamine isomers. Stereoselectivity becomes more evident (although the effect is not linear) upon increasing the substituent size from a methyl to an isopropyl group (Beckett and Brookes, 1970; Vree et al., 1971b). With propylamphetamines the R-(-)-enantiomers are preferentially dealkylated while total loss of stereoselectivity was observed with butylamphetamine enantiomers. On the basis of _in vitro_ studies with rat liver microsomes, it was suggested that differences in the activation energy requirements for S-(+)- and R-(-)-isopropylamphetamine accounted for their differential metabolism (Henderson et al., 1974). In N-alkylated amphetamines (N-methyl, -ethyl, and -isopropyl) replacement of the α-hydrogen with deuterium resulted in a marked increase in the activation energies of the S-(+)-isomers whereas isotopic effects were not evident with the R-(-)-enantiomers (Vree et al., 1971a, 1971b; Henderson et al., 1974). These results suggested that in the dealkylation and deamination of S-(+)-secondary amphetamines, removal of the proton on the α-carbon was the rate-limiting step. In contrast, oxidation of the R-(-)-isomers proceeds via N-oxidation. Stereoselective metabolic effects were not observed with tertiary amphetamines (Reference number 27 in Jenner and Testa, 1973).

N-Dealkylation of N,N-dialkylamphetamines via N-oxidation is the most obvious metabolic route. The calculated apparent activation energies for N-dealkylation of N,N-dimethylphentermine and N,N-dimethylphenylethylamine were found to be 17.5 +/- 1.5 and 17.7 +/- 0.05 Kcal/mole, which correspond to an N-oxidation process (Hendersen et al., 1974). In another study (Beckett et al., 1978) it was observed that the amount of S-(+)-amphetamine formed after incubation of S-(+)-N-benzylamphetamine with rat and rabbit liver microsomal preparations was almost double that obtained from the R-(-)-isomer. The products of N-oxidation were predominantly selective for R-(-)-benzylamphetamine. Interestingly, the percentage of unmetabolized substrate was identical for both isomers.

It is evident that the species of animal affects metabolic stereoselectivity. Although in vivo (Morgan et al., 1972) and in vitro (Reference number 25, Jenner and Testa, 1976) experiments in rats have demonstrated stereoselective demethylation of S-(+)-N-methylamphetamine, Beckett and Haya (1978) observed that with rabbit hepatic fractions, R-(-)-N-ethylamphetamine was the preferred substrate for both N-dealkylation and N-oxidation. In humans, S-(+)-ethylamphetamine is selectively N-dealkylated (Vree et al., 1971a, 1971b).

Species differences are also observed in the metabolism of fenfluramine. The S-(+)-enantiomer is preferentially N-deethylated in humans (Brooks and Beckett, 1970) while the reverse is true in rats (Morgan et al., 1972). The metabolic similarity with (+/-)-ethylamphetamine should not be surprising. However, the introduction of m-CF_3 into ethylamphetamine substantially reduces the stereoselective metabolism in humans (Beckett and Brooks, 1970). The difference in the stereoselectivity of distribution of fenfluramine in rat plasma, striatum (site of action for anorectic agents), brain stem and erythrocytes is similar (Jori et al., 1978). Intraperitoneal administration of fenfluramine to rats resulted in higher levels of S-(+)-isomers than R-(-)-isomers and concomitantly there was more

R-(-)-norfenfluramine than the S-(+)-isomer. These differences were not related to stereoselective absorption from the peritoneal cavity as similar observations were made following intravenous administration. Rapid disposition of R-(-)-fenfluramine is believed to be of metabolic origin as its elimination was completely suppressed when the liver microsomal inhibitor SKF 525 was administered. This in all probability also suggests the involvement of different enzyme systems in the process of dealkylation (Jenner and Testa, 1973). The observations on fenfluramine were confirmed in a separate study that investigated the kinetics of the isomers in rats (Caccia et al,, 1981). It was suggested that after oral administration to rats, the rate of fenfluramine absorption was similar for both isomers, while only small differences in their distribution rate constants were observed. The difference in the rates of hepatic metabolism results in preferential disappearance of R-(-)-fenfluramine, while more R-(-)-norfenfluramine accumulates than the corresponding S-(+)-isomer. Rates of disappearance of both fenfluramine isomers have been shown to be dose-dependent, decreasing disproportionately from lower to higher doses. The observed differences in the kinetic profiles of S-(+)- and R-(-)-fenfluramine in rats and mice are consistent with stereoselective N-deethylation of the R-(-)-isomer. In contrast there is little difference in the kinetic and metabolic profiles of the isomers in humans and dogs (Caccia et al., 1982).

Some recently reported studies dealing with drugs which undergo stereoselective N-dealkylations include: chlorpheniramine (Thomson and Shioshita, 1981), disopyramide (Cook et al., 1982), propranolol (Nelson and Bartels, 1984b), morphine (Rane et al., 1985) and a mechanism in reverse, stereoselective methylation of nicotine (Cundy et al., 1985).

1.3.3.0.0.0 MAMMALIAN CHIRAL INVERSION AND DISPOSITION OF 2-ARYLALKANOIC ACIDS

The stereoselective disposition of chiro-optical arylalkanoic acids, particularly the 2-arylpropionic acids (APAs), has been a subject of recent interest. Of particular intrigue is the unidirectional metabolic optical inversion of the distomer (l-, generally R-configuration) to eutomer (d-, generally S-configuration) (Hutt and Caldwell, 1983). In addition to chiral inversion, stereoselectivity in the pharmacokinetics of arylalkanoic acids may also arise due to selectivity in absorption, distribution, biotransformation (other than chiral inversion) and excretion.

In this section the issue of stereoselectivity of arylalkanoic acids, particularly chiral inversion, is discussed along with appropriate references to other factors affecting stereoselectivity.

The chiral inversion of APAs is a unique aspect of the metabolic behaviour of an important group of non-steroidal antiinflammatory drugs. The clinical significance of the bioinversion of distomer to eutomer has recently been emphasized (Hutt and Caldwell, 1983, 1984, 1985; Ariens, 1984; Williams and Lee, 1985), although it was first mentioned in a survey of novel metabolic routes (Israili et al., 1977). These in vivo optical inversions are characteristically unidirectional and always favour the formation of the dextro isomer. The antiinflammatory properties are mainly due to the S-(+)-isomer (Harrison et al., 1970; Nickander et al., 1971; Tomlinson et al., 1972; Ku and Waswary, 1975; Greig and Griffin, 1975; Adams et al., 1976; Buttinoni et al., 1983; Rubin et al., 1985), however, as a result of inversion the two isomers may approach bioequivalence in vivo (Adams et al., 1976).

Chiral inversion is not the only factor that determines the relative pharmacokinetic and pharmacological responses of the enantiomers. There are many other processes that affect the enantiomeric composition of a racemic drug in the body, including absorption, distribution, metabolism (other than inversion) and excretion (Jenner and Testa, 1973; Low and Castagnoli, 1979).

Chiral inversion has been illustrated for ibuprofen (Wechter et al., 1974; Kaiser et al., 1976; Lee et al., 1985; Cox et al., 1985), fenoprofen (Rubin et al., 1985), benoxaprofen (McKay et al., 1979; Kemmerer et al., 1979; Simmonds et al., 1980), cicloprofen (Lan et al., 1976; Kripalani et al., 1976), 2-(2-isopropylindan-5-yl)-propionic acid (Tanaka and Hayashi, 1980), naproxen (Goto et al., 1980), clidanac (Tamura et al., 1981) and hydratropic acid (Yamaguchi et al., 1985). Although bioinversion has been described as a general phenomenon (Hutt and Caldwell, 1983), some arylpropionic acids such as indoprofen (Tosolini et al., 1974; Bjorkman, 1985) and possibly carprofen (Stoltenborg, 1981) appear to be exceptions.

Several mechanisms based on isotope labelling studies have been advanced to explain the inversion process. The mechanism proposed by Wechter and co-workers (1976) was conceptualized on the basis that the APAs were incorporated into triglycerides; this was later confirmed with fenoprofen and ketoprofen (Fears et al., 1978). Wechter proposed the existence of an R-arylpropionic acid isomerase (R-APAI) enzyme operating within the lipid catabolism and anabolism pathways (Fig. 7). It was observed that after administration of tetradeuterated R-(-)-ibuprofen to human subjects, the S-(+)-ibuprofen recovered from biological samples had retained only two deuterium atoms on the methyl portion of the drug (Fig. 8). Furthermore, it was determined that the S-isomer was not a substrate for the proposed isomerase enzyme, i.e. there was no S- to R-inversion.

This concept, which predicts the formation of a methylene intermediate by a dehydrogenase enzyme has been contradicted by other investigators. *In vitro* rat liver subcellular incubations with racemic α-deutero (methine - d$_1$) ibuprofen and α-CD$_3$-ibuprofen (Nakamura et al., 1981) suggested that inversion was mediated by abstraction of the methine proton. These observations were reproduced by means of perfused rat liver experiments, thus suggesting alternate pathways mediated by ATP and coenzyme A. Isolation of R-(-)-ibuprofenyl CoA from rat liver homogenates and the observation that synthetic S-(+)-ibuprofenyl CoA inverts *in vivo* suggested that coenzyme A showed stereoselective activity and that R-(-)-ibuprofenyl CoA was epimerized by the nonspecific isomerase enzyme (Nakamura et al., 1981) (Fig. 9). These studies were confirmed using labelled 2-(2-isopropylindan-5-yl)propionic acid (Tanaka and Hayashi, 1980) and clidanac (Tamura et al., 1981).

It is difficult to explain the observed differences between the proposed inversion mechanisms; species variation and, in some instances, drug differences may play a role. While investigating the inversion of cicloprofen in rats and monkeys, Lan and co-workers (1976) proposed various inversion pathways (Fig. 10). Their preliminary evidence favored the formation of a methylene intermediate as this substrate was readily converted to cicloprofen.

The various pathways have been summarized best by Lee and co-workers (1985) (Fig. 11). The prerequisite for inversion appears to be the highly stereoselective esterification of the R-(-)- isomer to its coenzyme A thioester. Once the ester is formed, subsequent epimerization occurs by means of an enzyme similar to methylmalonyl coenzyme A racemase. In Figure 11 the process of metabolic inversion is depicted in the proper perspective, i.e. the inversion process is competitive with other routes of metabolism.

There is limited evidence suggesting that inversion occurs sometime during or after absorption and that it is terminated after extraction by the liver. _In vitro_ rat liver (perfused and supernatant fraction) incubations containing ibuprofen (Nakamura et al., 1981; Cox et al., 1985) and benoxaprofen (Simmonds et al., 1980) as well as incubates using inverted rat intestine and benoxaprofen (Simmonds et al., 1980) all resulted in substantial inversion of the respective substrates.

Animal species vary in their ability to invert APAs. In a comparative study, rats and dogs were shown to be excellent inverters of cicloprofen while monkeys, like humans, were poor inverters (Lan et al., 1976). These observations indirectly reflect the significance of the mesenteric circulation in the unidirectional bioinversion of APAs. It is an interesting and important coincidence that the APAs not only undergo extensive inversion in rats but they also undergo significant biliary excretion. In contrast to these animals, humans are poor inverters, a fact which may be associated, in part, with their less pronounced biliary excretion.

It has also been reported that the extent of inversion of R-(-)-cicloprofen in female monkeys is greater than that in males (Lan et al., 1976). However as the study was done in a statistically insignificant population these results must be interpreted with some caution.

Humans with alcohol-induced cirrhotic livers as well as rats with fatty acid livers were found to be poor inverters of R-(-)-ibuprofen (Cox et al., 1985). This suggests that a normal healthy liver plays a role in the inversion process.

In humans, the APAs are quickly absorbed, highly protein bound, and rapidly excreted in the urine with short elimination half-lives (Verbeeck et al., 1983). A short elimination half-life has an apparent negative effect on inversion; benoxaprofen has an inversion half-life (about 108 hours) longer than the elimination half-life (about 30 hours) and undergoes insignificant inversion in humans (Simmonds et al., 1980).

Overall stereoselectivity in the disposition of APAs may also occur due to differences in the metabolism and excretion of the enantiomers. The exact role of biotransformation is not entirely clear. With the exception of naproxen (Verbeeck et al., 1983), the APAs, e.g. indoprofen (Fucella et al., 1973), carprofen (Rubio et al., 1980), and tiaprofenic acid (Pottier et al., 1977; Sorkin and Brogden, 1985; Jamali et al., 1985), undergo little Phase I biotransformation.

The major cause of enantiomer-associated discrimination in the disposition of indoprofen (Tosolini et al., 1974; Buttinoni et al., 1983; Tammassia et al., 1984; Bjorkman, 1985) and possibly carprofen (Stoltenborg et al., 1981) is differences in the excretion rates of the isomers. This, in turn, arises from stereoselective protein binding. In this respect, stereoselective disposition of (R,S)-hydratropic acid could be of particular interest (Yamaguchi and Nakamura, 1985) because almost all of the APAs are considered to be derivatives of this compound. In rats the plasma protein binding of hydratropic acid is the most important factor controlling the disposition of this drug. This illustrates that apart from chiral inversion, stereoselectivity could also arise from other alternative pathways.

In summary, it appears that the unidirectional inversion of APAs is a metabolic process which, like any other biotransformation, is influenced by competing metabolic pathways. Animal species, sex, and the presence of a disease state may also influence the stereoselective disposition of APAs.

As part of the thesis research, two NSAIDs of particular interest were tiaprofenic acid (d,l-5-benzoyl-α-methyl-2-thiopheneacetic acid) and etodolac or etodolic acid (1,8-diethyl-1,3,4,9-tetrahydropyrano{3,4-b}indole-1-acetic acid). These NSAIDs are of recent origin and are still under clinical trial in North America. Tiaprofenic acid is a typical APA except that the carboxylate group is attached to a heterocyclic thiophene rather than phenyl ring. The drug is effective in suppressing inflammation in standard animal models (Fujimura et al., 1975; Deraedt et al., 1980, 1982) and is more potent than indomethacin and ibuprofen. However, it tends to be less effective in acute and chronic adjuvant-induced arthritis (Deraedt, 1982). As an analgesic it is more potent than aspirin and phenylbutazone (Deraedt, 1980), equipotent with diclofenac and ibuprofen, and less potent than ketoprofen or indomethacin (Deraedt et al., 1982). In humans, therapeutically equivalent doses of tiaprofenic acid produce fewer signs of gastric irritation than indomethacin or aspirin (Lucker et al., 1982).

Non-stereoselective pharmacokinetic studies have determined that racemic tiaprofenic acid is readily absorbed after oral administration, with peak plasma concentration (around 30 mg/L) attained within 2-3 hours of dosing (Daymont and Herbert, 1981, 1982, 1983; Pottier et al., 1982; Jamali et al., 1985). Absorption is influenced by food (Sorkin and Brogden, 1985) and route of administration (Pottier et al., 1982).

The racemic drug and its metabolites are distributed throughout the body in proportion to the blood concentration of tiaprofenic acid, suggesting that rapid absorption takes place (Kaneto and Matsui, 1977). The volume of distribution of tiaprofenic acid in humans is close to the plasma volume and ranges from 4-10.6% of body weight (Pottier et al., 1977, 1982; Jamali et al., 1985). Tiaprofenic acid diffuses well into synovial fluid; equilibrium is achieved within 4 hours of administration after which time the plasma concentrations are lower than the synovial concentrations. The preceeding observation is in agreement with the behaviour of other NSAIDs (Wallis and Simkin, 1983) and is regarded as a positive aspect to the therapeutic action of tiaprofenic acid in an inflamed joint (Honig et al., 1984).

Tiaprofenic acid undergoes limited Phase I biotransformation to phenolic (oxidative) and alcoholic (reductive) metabolites (Nahoul, 1979; Pottier et al., 1977, 1982; Jamali et al., 1985). The drug is completely eliminated from the body mainly as urinary acyl conjugates (Lucker et al., 1977; Pottier, 1977, 1982) within 24 hours. Trace amounts are found in plasma after this period (Honig, 1984).

Pharmacokinetic studies on the disposition of tiaprofenic acid in healthy subjects and arthritic patients with renal dysfunction (Sorkin & Brogden, 1985) have revealed that a higher volume of distribution and prolonged elimination half-life occurs in patients with renal dysfuntion. However, the pharmacokinetics of the drug in an arthritic patient are not significantly different from those in a normal subject (Jamali et al., 1985).

Etodolac (Demerson et al., 1984) has shown potent analgesic and antiinflammatory properties in laboratory animals (Demerson, 1976). It is approximately 6 times more potent than phenylbutazone in depressing adjuvant arthritis and equipotent in carageenan paw edema and analgesic studies (Martel and Klicius, 1976). In humans, etodolac is effective in the treatment of post-surgical pain

(Verischelen, 1982; Gaston et al., 1984; Fliedner et al., 1984), episiotomy pain (Freidrich, 1983), and rheumatoid and osteoarthritis (Vetter et al., 1982; Gordon et al., 1983; Ryder et al., 1983; Sanda et al., 1983; Jacob et al., 1985). It is superior to ASA (Verischelen et al., 1982; Andelman, 1983; del-Toro et al., 1983; Edwards, 1983; Friedrich, 1983; Gordon and Polsky, 1983; Jacob et al., 1983) and is better tolerated in the dosage range of 50-500 mg/day with a significantly lower incidence of gastrointestinal-related side effects (vide infra). Even though the ulcerogenic/therapeutic ratio is more favorable than that of phenylbutazone and indomethacin, etodolac is slightly more ulcerogenic than phenylbutazone but less than indomethacin (Martel et al., 1976). The drug is associated with a low incidence of gastrointestinal bleeding (Joubert et al., 1982; Arnold et al., 1984; Salom et al., 1984) and is capable of reversing skeletal changes associated with arthritis (Martel et al., 1984; Anon, 1985).

Etodolac is an arylacetic rather than arylpropionic acid. It further differs from other NSAIDs in that the plasma protein binding is less (about 25% with serum concentration of 100 ug/mL), it does not depend upon ester conjugation for its elimination (Cayen et al., 1981) and it has an elimination half-life of approximately 7 hours (compared to 2 hours for APAs) (Cayen et al., 1981; Cosyns et al., 1983; Kraml et al., 1984). The drug is quickly absorbed, with a linear relationship observed between dose and AUC.

Three Phase I metabolites have been recently identified in humans (Ferdinandi et al., 1986). The metabolites 7-hydroxyetodolac (major), 6-hydroxyetodolac and 8-(hydroxyethyl)etodolac were isolated as glucuronyl ester conjugates from urine.

Studies involving _in_ _vitro_ inhibition of prostaglandin synthetase reveal that the d-isomer of etodolac is relatively more active than the l-isomer (Demerson et al., 1983, 1985).

2.0.0.0.0.0 MATERIALS AND METHODS

In the following section is described the experimental protocol including the dosing of various biological models (microbial, animal and human) with different substrates (a β-lactam, a β-adrenoceptor agonist, amphetamine analogs, and non-steroidal antiinflammatory drugs), and the collection of various sample specimens (culture media, cell mycelia, rat brain or liver tissue and human plasma, synovial fluid or urine) for their chromatographic analysis (stereospecific or non-stereospecific GC and HPLC). Each concentration value thus obtained represents the mean of two readings (i.e. duplicate samples).

2.1.0.0.0.0 CHEMICALS AND REAGENTS

Chemicals, reagents and their sources are listed below. Routinely used solvents were purchased from local vendors (Fisher Scientific and Terochem Laboratories, Edmonton Alberta) and were glass-distilled prior to use. Water was deionized and distilled using a Milli-Q Reagent Water System (Millipore, Bedford, MA). All standard solutions were stored at 4°C.

R,S-, R-, S-Amphetamine (Health and Welfare Canada, Ottawa, Ontario)

Bacto Sabouraud Dextrose Broth, dehydrated; Czapek-Dox Broth, dehydrated (Difco Laboratories, Detroit MI)

4-Benzyloxyphenol (Lancaster Synthesis, Windham, NH)

(-)-Camphanic acid chloride; 1,1'-carbonyldiimidazole; dicyclohexylcarbodiimide; epichlorhydrin; 1-hydroxybenzotriazole hydrate; isopropylamine; isopropyl alcohol; R-(-)- and S-(+)-α-methylbenzylamine; phenol; thionyl chloride; triethylamine (Aldrich Chemical Co., Milwaukee, WI)

Magnesium sulfate; perchloric acid; sodium carbonate; sodium bicarbonate; sodium chloride (Fisher Scientific, Edmonton, Alberta)

Chloroform-d (MSD Isotopes, Montreal, Quebec)

Di-(2-ethylhexyl)phosphate (Sigma Chemical Co., St. Louis, MO)

Trifluoroacetic anhydride (Pierce Chemical Co., Rockford, IL)

N-Trifluoroacetyl-L-prolyl choride (Regis Chemical Co., Morton Grove, IL)

Silica gel (Camag, Hamburg, FRG)

R,S-Cicloprofen (E.R. Squibb, Princeton, NJ)

(+/-)-, (+)-, (-)-Etodolac; R,S-propranolol (Ayerst, New York, NY)

R,S-Fenoprofen (Eli Lilly, Indianapolis, IN)

R,S-Flurbiprofen (Boots, Nottingham, U.K.)

R,S-Ibuprofen (Upjohn, Don Mills, Ontario)

R,S-, S-Ketoprofen (Rhone-Poulenc, France)

S-Naproxen (Syntex, Palo Alto, CA)

R,S-Pirprofen (Ciba-Geigy, Basle Switzerland)

R,S-Tiaprofenic acid (Roussel, Montreal, Quebec)

R,S-Benzylamphetamine; R,S-*n*-butylamphetamine; R,S-*p*-chloro- fluoroamphetamine; R,S-fenfluramine; R,S-fluoroamphetamine; R,S-fluorotranylcypromine; R,S-isopropylamphetamine; R,S-norfenfluramine; R,S-methylamphetamine (synthesized in the laboratories of Dr. R.T. Coutts, Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta)

N-Methyletodolac (synthesized by Dr. L. Chu, Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta) 7-[(Phenoxy)acetamido]desacetylcephalosporanic acid, methyl (CS) and ethyl esters, and R-sulfoxide of methyl ester (synthesized in the laboratories of Dr. R. Micetich, Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta)

2.2.0.0.0.0 GLASSWARE

All experiments were conducted in reusable borosilicate glassware. The test tubes were in two sizes; 16 x 125 mm and 13 x 100 mm. Tubes could be sealed using plastic caps with Teflon-faced rubber liners. The microbial cultures were incubated in 125 mL Erlenmeyer flasks.

All glassware was thoroughly washed with a commercial glassware detergent immediately after use. This was followed by thorough rinsing with distilled water and methanol. After drying at room temperature the glassware was heated in a muffle furnace at 400°C for at least 1 hour.

2.3.0.0.0.0 MISCELLANEOUS EQUIPMENT

The equipment (and suppliers) utilized routinely during the course of these studies are listed below.

Dissolution of substrates by ultrasonification - Bransonic Ultrasonicator (Branson, Shelton, CONN)

Sample vortexing - IKA Vibrax VXR Shaker (Janke and Kunkel, Staufen, FRG); Vortex Genie (Scientific Industries, Springfield, MA)

Centrifugation (low speed, 3000 rpm) - Adams Dynac Centrifuge (Clay Adams, Parsipanny, NJ)

Centrifugation (high speed, 10,000 rpm) - IEC B-20A Centrifuge, Damon/IEC Division, Needham Hts., MA)

Solvent evaporation and concentration - Savant Speed Vac Concentrator (Emerston Instruments, Scarborough, Ontario)

Sample heating - Reacti-Therm Heating Block (Pierce

Chemical Co., Rockford, IL)

Microorganism handling and culturing - Biogard Laminar Hood (Baker Co., Sanford, Maine)

Microbial incubations - G-25 Controlled Environment Incubator Shaker (New Brunswick Scientific, Edison, NJ)

Autoclave and sterilization - Amsco Steam Autoclave (Amsco, Erie, PA)

Tissue disruption and homogenization - Potter-Elvejheim Homogenizer with glass grinding tube, Teflon glass pestle and fixed speed motor (Bodine Electric Co., Chicago, IL)

Melting point determinations - Thomas Hoover Melting Point Apparatus (Arthur H. Thomas, Philadelphia, PA)

2.4.0.0.0.0 GC AND HPLC EQUIPMENT

The HPLC system (Waters Scientific, Mississauga, Ontario) consisted of a model 6000A pump, Wisp autosampler, model 481 variable wavelength (UV) detector, model 730 data module, a 10 cm reverse phase column (uBondapak C18, Waters Scientific) and a 5 cm guard column. The mobile phase, methanol:water:acetic acid (50:48:2) was passed through 45 um filters (Millipore, Mississauga, Ontario).

The GC was a Hewlett Packard model 5730A equipped with a nitrogen phosphorus detector or a model 5700A with a flame ionization detector. Both chromatographs had a 18740B capillary column controller and a 3390A integrator-recorder (Hewlett Packard, Palo Alto, CA).

The fused silica capillary column (12m x 0.2 mm I.D.) was coated (0.33 um film thickness) with a high performance cross-linked dimethylsilicone film (Hewlett Packard) and was used throughout the studies. The operating conditions, except for the column temperature, were consistently maintained at the following settings: injection port, (operated in the splitless mode) 250°C; detector, 300°C. The gas flow rates were: helium (carrier gas), 2 mL/min; hydrogen, 3 mL/min; air, 50 mL/min.

2.5.0.0.0.0 SPECTROMETERS

UV absorbance spectra of β-lactam derivatives were obtained on a Unicam SP-1800 UV spectrophotometer (Pye Unicam, Cambridge, U.K.). ^{1}H-NMR spectra were recorded on a Varian EM 390 (Varian Canada, London, Ontario) or Bruker AM-300 (Bruker Spectrospin Canada, Milton, Ontario) spectrometer. IR spectra were obtained on a Nicolet 5DX (Nicolet, Madison WI) spectrometer.

The structures of compounds derivatized for GC analysis were confirmed by EI and CI mass spectra (GC/MS) obtained on a Hewlett Packard 5980A or VG 7070E (Analytech Instrumentation and Service, St. Laurent, Quebec) instruments. High resolution mass spectra were obtained on an AEI MS-50 spectrometer (Department of Chemistry, University of Alberta).

2.6.0.0.0.0 BIOLOGICAL MODELS: SOURCE AND SUPERVISION

MICROORGANISMS:The microorganisms chosen for these studies were fungi obtained from the University of Alberta Mold Herbarium (Edmonton, Alberta) or the American Type Culture Collection (Rockville, MD).

ANIMALS: For animal studies male Sprague-Dawley rats were purchased from Bio-Science Animal Services (Ellerslie, Alberta). The animals were housed in plastic cages on cedar chip bedding, in a temperature controlled room and subjected to a 12 hour on/off lighting schedule. The rats were allowed access to food and water (ad lib) until the day of the experiment.

HUMAN SUBJECTS: Studies involving human subjects were conducted under the supervision of Dr. F. Jamali (Faculty of Pharmacy & Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta) in cooperation with Dr. A.S. Russell (Division of Rheumatology, Faculty of Medicine, University of Alberta) and the staff of Station 35, University of Alberta Hospital.

2.7.0.0.0 GENERAL MICROBIAL INCUBATION PROCEDURE: PREPARATION OF THE MEDIA

The dehydrated media were reconstituted with appropriate volumes of water; Czapek-Dox broth (35 g/L solution) and Bacto Sabouraud broth (30 g/L). The liquid media were then distributed (25 mL) into 125 mL Erlenmeyer flasks fitted with metallic caps. Media were autoclaved for 15 mins (121°C at 20 psi) prior to addition of the microorganism and filter-sterilized substrate.

2.7.1.0.0.0 METHODOLOGY FOR INCUBATION AND SAMPLE LOADING

A typical methodology for incubation and sample loading is depicted in Figure 14.

2.7.1.2.0.0 MICROBIAL SULFOXIDATION OF A β-LACTAM DERIVATIVE

2.7.1.2.1.0 INCUBATION AND SAMPLE COLLECTION

A number of different fungi were screened for sulfoxidation capability. Phase II cultures (25 mL) of Aspergillus alliaceus, Aspergillus terreus ATCC 20542, Cunninghamella echinulata UAMH 4145, Curvularia lunata UAMH 4378, Rhizopus nigricans, Trichoderma polysporum, and Trichoderma viride UAMH 4161T were spiked with 5 mg of 7-[(phenoxy)acetamido]desacetylcephalosporanic acid methyl ester (abbreviated CS) dissolved in a minumum amount of acetone (approximately 0.5 mL). To determine the presence of the oxidized substrate (i.e. the sulfoxide, abbreviated CSO) aliquots (1 mL) of the broth were monitored by HPLC, 24 hours post sample loading.

For investigating the kinetics of the rate of uptake of CS and the corresponding CSO formation, Phase II cultures of the selected organism, Cunninghamella echinulata UAMH 4145, were loaded with 0.5, 2.5, 5, and 10 mg per culture to give quantities of 25, 100, 200 and 400 ug/mL respectively. The cultures were monitored (HPLC) for CSO formation at appropriate intervals; cultures containing 200 and 400 ug/mL of substrate were monitored every 48 hours whereas 25 and 100 ug/mL cultures were monitored every 24 hours. Incubations were terminated one sampling interval after the disappearance of the substrate.

2.7.1.2.2.0 HPLC ANALYSIS OF THE CEPHALOSPORANIC ACID SULFOXIDE

The cell free culture medium (1 mL) was drawn at appropriate times and analyzed according to the protocol depicted in Figure 15.

For the purposes of preparing calibration curves, 1 mL portions of freshly prepared and autoclaved Czapek-Dox medium were spiked with 5, 10, 20, 40, 80, 100, 200, 400 and 600 ug of CS and authentic CSO (R-configuration) and 25 ug of the ethyl ester of CS as the internal standard.

2.7.1.2.3.0 SCALE-UP STUDY

For scale-up studies, 25 mL of homogenized Phase I cultureswere added to Czapek-Dox medium contained in a 2L-capacity airlift reactor. Oxygen was supplied at a rate sufficient to maintain circulation of the medium but low enough to prevent foaming and excessive evaporation. After 48 hours the well-developed microbial pellets were given 400 mg of CS. At the end of day 3 (72 hours of incubation) the medium was separated from the pellets (filtration) and the pH adjusted to 7.4 with sodium bicarbonate. The medium was then subjected to continuous extraction (liquid-liquid extractor) for 24 hours at room temperature using ethyl acetate:chloroform (40:60). The organic solvent was then dried over anhydrous sodium sulfate and concentrated under vacuum. After reconstituting with ethyl acetate (2 mL) the crude extract was purified by TLC.

2.7.1.2.4.0 THIN LAYER CHROMATOGRAPHY

A solution of the crude extract was applied as a thin continuous streak across 4 thin layer plates (200 x 200 cm glass plates coated with silica gel, 0.3 mm thick). The plates were then developed in a TLC chamber previously equilibrated with the solvent system, acetic acid:water:methanol (4:1:5). After development, the area of the plate corresponding to the metabolite CSO (determined by comparison with authentic CSO observed under UV light) was scraped off and extracted with two 10 mL portions of ethyl acetate:chloroform (4:6). The organic solvent was removed under a nitrogen stream and the dry residue reconstituted with deuterated chloroform (about 0.5 mL containing 1% tetramethylsilane) for ^{1}H-NMR analysis.

2.7.2.0.0.0 MICROBIAL SYNTHESIS OF RACEMIC PRENALTEROL

2.7.2.1.0.0.a CHEMICAL SYNTHESIS OF 1-PHENOXY-3-(ISOPROPYLAMINO)-2-PROPANOL (RACEMIC PREPRENALTEROL) AND ITS HYDROCHLORIDE SALT

Racemic pre-prenalterol (PP) was utilized as the precursor in the directed microbial synthesis of racemic prenalterol. It was synthesized according to a procedure reported for the adrenoceptor blocking agent metoprolol (Arfwidsson et al., 1976). Phenol (0.1 M) and epichlorhydrin (0.1 M) were refluxed with potassium carbonate (28 g) in acetonitrile (75 mL) for 5 hours. The inorganic salts were then removed by filtration and the organic solvent evaporated to dryness. After two co-evaporations with toluene, the crude epoxide (8.4 g) was used directly in the next reaction step. The epoxide, in isopropyl alcohol (30 mL), was refluxed with isopropylamine (0.5 M) for 3 hours. This gave the final product PP which was recovered by evaporation of the organic solvent and purified by recrystallization from acetonitrile. The ^{1}H-NMR, IR, and mass spectra were consistent with the expected product.

The hydrochloride salt was prepared by dissolving a portion of the free base in isopropyl alcohol and subsequent passage of HCl gas. The precipitated salt was collected by filtration and recrystallized from acetonitrile. Melting point, 107°C; reported (Zaagsma and Nauta, 1974), 110-112°C (ether-methanol).

2.7.2.1.0.0.b **CHEMICAL SYNTHESIS OF 1-(p-HYDROXYPHENOXY)-3-(ISOPROPYLAMINO)-2-PROPANOL (RACEMIC PRENALTEROL) AND ITS HYDROCHLORIDE SALT**

A standard sample of racemic prenalterol (P) required for authentication of the microbial product was synthesized according to a reported procedure (Crowther et al., 1969). The synthetic scheme was identical to that described for PP except that the starting material was 4-benzyloxyphenol. The 4-hydroxy function was deprotected by means of catalytic hydrogenation (5% Pd-C, 40 psi for 5 hours) to give racemic prenalterol. Physical data were consistent with the expected product.

A portion of prenalterol free base was converted into the hydrochloride salt in a manner similar to that described for PP. Melting point, 165°C (acetonitrile); reported (Crowther et al., 1969), 167-168°C (ethanol-ethyl acetate).

2.7.2.2.0.0 INCUBATION AND SAMPLE COLLECTION

The kinetics of microbial synthesis of racemic prenalterol was investigated after loading separate 25 mL Czapek-Dox Phase II cultures of *C. echinulata* with 2.9, 5.8 and 11.6 mg of PP hydrochloride (equivalent to 2.5, 5 and 10 mg of free base respectively). The cultures were then monitored for 8 days to determine the rate of uptake of PP and subsequent formation of prenalterol. Aliquot portions (25 uL) of the cell free culture medium were withdrawn from the cultures every 12 hours and subjected to GC analysis.

2.7.2.3.0.0 GC ANALYSIS OF PRE-PRENALTEROL AND PRENALTEROL

The method employed for simultaneous determination of PP and prenalterol was adapted from a reported GC/MS assay of prenalterol in human plasma and urine (Ervik et al., 1982).

2.7.2.3.1.0 STANDARD SOLUTIONS

Standard stock solutions containing prenalterol and PP hydrochlorides (equivalent to 1 mg/mL of free base) were prepared in distilled water and stored at 4°C. Test solutions (100 ug/mL in fresh Czapek-Dox medium) were prepared from the stock prior to assay. A standard solution (100 ug/mL in Czapek-Dox medium) of the internal standard (racemic propranolol) was prepared separately and stored at 4°C. Sodium carbonate buffer solution (pH 9.9) was prepared fresh daily by dissolving sodium carbonate (2M) in water (200 mL) and adjusting the pH to 9.9 with sodium bicarbonate.

2.7.2.3.2.0 EXTRACTION AND DERIVATIZATION

A 25 uL portion of cell-free medium, 20 uL of the propranolol solution and 200 uL of carbonate buffer were combined in a test tube. The mixed solutions were then extracted with a 3 mL portion of toluene. The residual aqueous layer was subsequently basified with 50 uL NaOH (1M) and extracted again with toluene (3 mL). The two organic extracts were combined and evaporated to total dryness. The dry residue was then reconstituted with ethyl acetate (50 uL) and trifluoroacetic anhydride (200 uL). The mixture was vortexed for 30 sec and immediately evaporated under vacuum. The final residue was reconstituted with toluene (200 uL), 1 uL of which was analysed by GC.

2.7.2.3.3.0 CALIBRATION CURVES

The calibration curves were obtained by spiking 100 uL portions of cell free Czapek-Dox medium (diluted with 250 uL of buffer) with known amounts of PP and prenalterol to obtain final concentrations of 0.125, 0.25, 0.5, 1, 2, 5, 10, and 20 ug/mL. Following the addition of 20 uL of internal standard solution, the derivatization procedure was carried out as described above.

2.7.2.3.4.0 GC CONDITIONS FOR ANALYSIS

GC analyses were performed under a programmed temperature run (100-230°C at 32°C/min) with nitrogen/phosphorus detection. The chromatographic peaks corresponding to substrate (PP), metabolite (prenalterol) and the internal standard (propranolol) were identified by GC/MS.

2.8.0.0.0.0 CHIRO-OPTICAL ANALYSIS OF RACEMIC α-METHYLBENZENEETHANAMINE AND RELATED AMINES BY GAS CHROMATOGRAPHY

Qualitative GC separation of (N-alkylated)-α-methylbenzeneethanamines (amphetamines) and related amine enantiomers was attempted after conversion into the respective diastereoisomeric amide derivatives.

2.8.1.0.0.0 RESOLUTION OF N-TRIFLUOROACETYL-L-PROLYL AMIDE DERIVATIVES

2.8.1.1.0.0.a STANDARD STOCK AND TEST SOLUTIONS

Standard solutions (100 ug/mL of free base) of either the sulfate or hydrochloride salt of racemic amphetamine, N-benzylamphetamine, N-_n_-butylamphetamine, _p_-chloroamphetamine, fenfluramine, fluoroamphetamine, norfenfluramine and tranylcypromine were prepared in distilled water and stored at 4°C. Similar standard solutions (100 ug/mL of free base) of the optically pure isomers of amphetamine, fluoroamphetamine and N-methylamphetamine were also prepared in water and stored at 4°C.

2.8.1.1.0.0.b DERIVATIZATION AND EXTRACTION

Distilled water (0.5 mL) was spiked with the racemic amine solutions (200 uL) and basified with 1M NaOH (50 uL) prior to extraction with diethyl ether:dichloromethane (60:40, 2 x 3 mL). The organic extract was evaporated to dryness and the residue reconstituted with dichloromethane

(1 mL). To this was added N-trifluoroacetyl-L-prolyl chloride (N-TPC) reagent (50 uL) and the solution shaken for 10 min after which triethylamine (50 uL) was added and the reaction allowed to proceed for another 10 mins. The reaction mixture was then washed successively with 1 mL each of 5N HCl and distilled water. After drying over anhydrous magnesium sulfate, the organic layer was evaporated to dryness. The residue was then reconstituted with toluene (100 uL), 2 uL of which was subjected to GC analysis.

2.8.1.1.0.0.c GAS CHROMATOGRAPHIC CONDITIONS

Resolution of the derivatized amines was achieved with the GC column maintained at 160°C using flame ionization detection. The structures of derivatized amphetamine, methylamphetamine, fluoroamphetamine, fenfluramine and norfenfluramine were confirmed by GC/MS.

2.8.1.2.0.0 DETERMINATION OF THE DIASTEREOISOMER ELUTION ORDER

The order of elution of the diastereoisomeric amides was determined by comparing the retention times of the diastereoisomers prepared from the racemates to those obtained from derivatives of the pure enantiomers when injected separately.

2.8.2.0.0.0 REACTION OF (-)-CAMPHANIC ACID CHLORIDE WITH (R,S)-AMPHETAMINE

2.8.2.1.0.0.a STANDARD STOCK AND TEST SOLUTIONS

Standard stock solutions of racemic amphetamine and the enantiomers were prepared as described above. A 0.01M solution of (-)-camphanic acid chloride was prepared by dissolving the compound (216 mg) in sufficient chloroform to give 100 mL of solution.

2.8.2.1.0.0.b EXTRACTION AND DERIVATIZATION

The steps involved in the extraction and derivatization of amphetamine (racemate and optical isomers) from test solutions were identical to those described for N-TPC amide derivatives.

2.8.2.1.0.0.c GAS CHROMATOGRAPHIC CONDITIONS

Aliquots (2 uL) of the final reconstituted solutions were injected into the GC (FID detection) under isothermal

(160°C) conditions. The structures of the final derivatives were confirmed by GC/MS.

2.8.3.0.0.0 STUDY OF THE STEREOSELECTIVE N-DEETHYLATION OF FENFLURAMINE IN RATS

2.8.3.1.0.0 DRUG ADMINISTRATION AND SAMPLE COLLECTION

Each experimental set consisted of 6 Sprague-Dawley male rats (180-220 g) dosed with racemic fenfluramine (40 mg/kg in saline) via intraperitoneal administration. A single rat was similarly injected with saline to act as a control. The rats were sacrificed by cervical dislocation at specified times coincident with the actual sample collection times, 0, 60, 120, 180, 240 and 300 mins after drug administration. Brain (without the pineal gland) and liver were excised from the surrounding tissues. Blood vessels surrounding the liver were removed immediately and the organs rinsed thoroughly with ice-cold saline solution. The washed tissues were then frozen solid by dipping into a mixture of isopentane/dry ice. The frozen tissues were then stored at -20°C until analyzed.

The experiment was repeated for 3 additional sets.

2.8.3.2.0.0 GAS CHROMATOGRAPHIC ANALYSIS OF FENFLURAMINE AND NORFENFLURAMINE ISOMERS IN RAT BRAIN AND LIVER TISSUES

2.8.3.2.1.0.a EXTRACTION AND DERIVATIZATION

The weighed tissues (brain or liver) were homogenized with 5 volumes of 0.4M perchloric acid and a portion (2 mL) of the homogenate retained for analysis. The homogenate was transferred into 15 mL centrifuge tubes and spiked with R-(-)-fluoroamphetamine (2 ug) as internal standard. The supernatant was then separated from the tissue by high speed centrifugation (10,000 rpm, 10 min at 4°C) and subsequently neutralized with solid $KHCO_3$. The solid precipitate of potassium perchlorate was separated by centrifugation (3,000 rpm, 10 min at room temperature) and the supernatant shaken (5 min) with 5 mL of the ion-pairing compound DEHP (2.5% v/v in chloroform). After centrifugation (3,000 rpm, 10 min at room temperature) the top aqueous layer was discarded. To the organic layer was added 0.1M HCl (3 mL) and the mixture vortexed for 5 mins. After centrifugal (3,000 rpm, 10 mins, room temperature) separation, the upper aqueous layer was retained for analysis. The acid layer was basified with 5M NaOH (0.5 mL) and extracted twice with 4 mL portions of toluene. The combined organic extracts were evaporated to complete dryness under vacuum. The residues were derivatized with N-TPC in a manner similar to that described earlier in Section 2.8.1.1.0.0.b .

2.8.3.2.1.0.b CALIBRATION CURVES

For the preparation of calibration curves, 0.4M $HClO_4$ tissue homogenates (2 mL) from control rats were spiked with known amounts of racemic fenfluramine, norfenfluramine and the internal standard R-(-)-fluoroamphetamine (2 ug).

The final concentrations obtained were 0.25, 0.5, 1, 2, 5, 10 and 20 ug/mL of racemic fenfluramine and norfenfluramine.

2.8.3.2.1.0.c GAS CHROMATOGRAPHIC CONDITIONS

Residues of the N-TPC derivatives of fenfluramine, norfenfluramine and the internal standard were reconstituted with toluene (200 uL) and the final solution (1 uL) subjected to GC-NPD analysis.

The column temperature was programmed from 100-250°C at 16°C/min; the initial temperature was held for 2 mins.

2.9.0.0.0.0 CHIRO-OPTICAL ANALYSIS OF RACEMIC ARYLALKANOIC ACIDS (NSAIDs) BY GAS CHROMATOGRAPHY

2.9.1.0.0.0.a STANDARD STOCK AND TEST SOLUTIONS

Standard stock solutions (1 mg/mL) of the NSAID arylalkanoic acids cicloprofen, etodolac (racemate and pure optical isomers), fenoprofen, flurbiprofen, ibuprofen, ketoprofen (racemate and the S-isomer), naproxen (S-isomer), pirprofen and tiaprofenic acid were prepared in methanol and stored at 4°C. Test solutions (100 ug/mL) were prepared from stock prior to derivatization. Standard solutions of 1,1'-carbonyldiimidazole, CDI (100 ug/mL and 65 mg/mL) and dicyclohexylcarbodiimide, DCC (100 ug/mL), were prepared fresh daily in methylene chloride or chloroform and solutions of R- and S-amphetamine sulfate (equivalent to 500 ug/mL of free base) were prepared in water and stored at 4°C.

2.9.1.0.0.0.b GC CONDITIONS FOR THE RESOLUTION OF NSAID DIASTEREOISOMERS

The same GC conditions were used for resolving the diastereoisomers prepared with optically pure α-methylbenzylamine or α-methylbenzeneethanamine. With the exception of ibuprofen (100-220°C at 16°C/min) the programmed oven temperature was 100-270°C at 32°C/min.

2.9.1.1.0.0 PREPARATION OF THIONYL CHLORIDE-MEDIATED α-METHYLBENZENEETHANAMINE DERIVATIVES OF IBUPROFEN, FENOPROFEN AND TIAPROFENIC ACID

Test solutions (250 uL) of the NSAIDs were added to pH 7 phosphate buffer (750 uL) and acidified to pH 1 with 5M HCl (100 uL). The solutions were then extracted with toluene (4 mL) and the organic extract evaporated to dryness. The residue was reconstituted with dichloromethane (1 mL) and to this solution was added thionyl chloride (50 uL). The tubes were screw-capped (sealed under nitrogen) and heated (85°C) for 30 mins. After allowing the reaction mixture to cool to room temperature the excess thionyl chloride was removed by evaporation in vacuo.

A 100 uL portion of S-(+)- or R-(-)-amphetamine sulfate solution was basified with 1M NaOH (100 uL) and extracted with toluene (4 mL). The amphetamine extract was added to the tubes containing the 3 NSAID acid chlorides prepared above. The mixture was vortexed (5 min) prior to the removal of solvent. The dry residue was reconstituted with methylene chloride (1 mL) and after vortexing (15 min) the solvent was washed successively with 1 mL portions of 1M HCl and distilled water. The washed solvent was then dried over magnesium sulfate and removed by evaporation.

The diastereoisomeric amide residue was then reconstituted with toluene (100 uL), 1 uL of which was subjected to analysis by GC-FID.

2.9.1.2.0.0 PREPARATION OF α-METHYLBENZENEETHAMINE DERIVATIVES OF ARYLALKANOIC ACIDS VIA CONDENSATION WITH 1,1'-CARBONYLDIIMIDAZOLE OR 1,3-DICYCLOHEXYLCARBODIIMIDE

Phosphate buffer (pH 7, 1 mL) was spiked with test solutions (100 uL) containing the NSAIDs, then acidified to pH 1 with 5M HCl (0.1 mL) and extracted with toluene (4 mL). A 100 uL portion of S-(+)- or R-(-)-amphetamine sulfate solution was basified with 1M NaOH (100 uL) and extracted with toluene (4 mL). The two extracts were combined in 13 x 100 mm glass tubes and evaporated to dryness. Each residue was reconstituted in chloroform or methylene chloride (1 mL) containing CDI stock solution (100 uL of a 100 ug/mL solution). The tubes were screw-capped (sealed under nitrogen) and heated (85°C) for 0.5, 1, 2 and 3 hours in the Reacti-Therm system or vortexed at room temperature (30 mins). After heating, the tubes were allowed to cool to room temperature and the reaction mixture was washed with 1M HCl (1 mL). The organic layer was dried over magnesium sulfate and evaporated to dryness. Each residue was reconstituted with toluene (100 uL), 1 uL of which was analyzed by GC with nitrogen phosphorus detection (GC-NPD).

Instead of using CDI, the above procedure was repeated with an equivalent amount of DCC. In this case the selected NSAID was ibuprofen and the coupling reaction was conducted at room temperature.

2.9.1.3.0.0 PREPARATION OF α-METHYLBENZYLAMINE DERIVATIVES OF IBUPROFEN AND FENOPROFEN USING THE METHOD OF VANGIESSEN AND KAISER (1975)

In order to evaluate the usefulness of optically active amphetamine as a resolving agent it was decided to compare it to optically active α-methylbenzylamine, the reagent most frequently utilized for the separation of NSAID enantiomers.

For this purpose, the method of Vangiessen and Kaiser (1975) was duplicated without any modification. The CDI reagent (0.1 mL of a 65 mg/mL solution) was added to tubes containing extracted, dry residues of ibuprofen and fenoprofen (10 ug) and then vortexed for 5 mins. After addition of acetic acid (10 uL) and R-(+)-α-methylbenzylamine (50 uL), the solution was vortexed for 20 mins). The solvent and excess reagents were removed by evaporation and the residue reconstituted with toluene (100 uL), 1 uL of which was analyzed by GC. The conditions for GC analysis were similar to those reported previously for ibuprofen-benzeneethanamine derivatives.

2.9.1.4.0.0 PREPARATION OF α-METHYLBENZYLAMINE DERIVATIVES OF IBUPROFEN AND FENOPROFEN USING THE α-METHYLBENZENEETHANAMINE PROTOCOL

The extracted dry residues of ibuprofen and fenoprofen (10 ug) were reconstituted with methylene chloride (1 mL). CDI (100 uL of a 100 ug/mL solution) and R-(+)-α-methylbenzylamine (50 uL) reagents were then added. One set of samples was allowed to react at room temperature (30 mins and vortexing) while the other was heated (85°C, 3 hours) in sealed tubes. Each reaction mixture was then washed successively with 1M HCl (1 mL) and water (1 mL). Each organic layer was dried over magnesium sulfate, evaporated to dryness and the residue reconstituted with toluene (100 uL), 1 uL of which was analyzed by GC-NPD.

2.9.2.0.0.0 STUDY OF THE STEREOSELECTIVE DISPOSITION OF ARYLALKANOIC ACIDS IN HUMAN SUBJECTS

2.9.2.1.0.0 GENERAL PROCEDURE FOR EXTRACTION AND DERIVATIZATION OF BIOLOGICAL SAMPLES

Suitable volumes of plasma (250-500 uL) or synovial fluid (500 uL), obtained from subjects dosed with an NSAID, were diluted with appropriate quantities of pH 7 phosphate buffer to give a final volume of 1 mL. These samples were then spiked with a suitable internal standard. After thorough mixing the solutions were basified with 1M NaOH (50 uL) and then extracted with diethyl ether (1 mL). The organic phase was discarded; the aqueous layer acidified with 5M HCl (0.1 mL) and extracted with toluene (5 mL). The urine samples (250-500 uL) were processed in a similar manner except that they were basified with 1M NaOH (50 uL) prior to dilution with phosphate buffer to facilitate extraction of basic impurities and hydrolyze any drug conjugates in the urine (Jamali et al., 1984, 1985). The 5 mL toluene extracts thus obtained constituted the drug sample.

Water (1 mL) was spiked with S-(+)-amphetamine sulfate stock solution (50 uL), basified with solid sodium bicarbonate or 1M NaOH (50 uL) and extracted with toluene

(5 mL). This extract was combined with the drug sample extract prepared above, and the solvent was evaporated to dryness. Each residue was reconstituted with dichloromethane (1 mL) to which was added CDI (200 uL of a 100 ug/mL solution). The tubes were purged with nitrogen, sealed, and heated (85°C) for 2 hours, with the exception of tiaprofenic acid which was heated for 1.5 hours at 65°C. The remainder of the procedure was as described previously for the test solution (Section 2.9.1.2.0.0).

2.9.2.2.0.0 A PRELIMINARY STUDY OF THE STEREOSELECTIVE DISPOSITION OF R,S-IBUPROFEN IN HUMAN SUBJECTS

This study was undertaken to determine the applicability of the procedures developed and discussed in the preceeding Sections to the determination of the stereoselective disposition of ibuprofen in human subjects.

2.9.2.2.1.0 DRUG ADMINISTRATION AND SAMPLE COLLECTION

After an overnight fast, nine male subjects were dosed with 600 mg ibuprofen (Motrin, Upjohn). The blood samples were collected at 0, 0.5, 1, 2, 3, 4, 6, 8 and 12 hours. The venous blood (4-5 mL) was drawn into heparinized

Vacutainer , tubes, the plasma separated by centrifugation (8,000 rpm, 10 mins) and the specimens stored at -20°C until analyzed. The cumulative urinary excretion was determined from total urine output (0-12 hours).

After a one-week washout period, subject no. 9 was given 600 mg of Apo-Ibuprofen. Blood and urine samples were collected as described for Motrin.

2.9.2.2.2.0 CALIBRATION CURVES FOR IBUPROFEN

The blank plasma or urine samples (0.5 mL) were diluted with an equal volume of pH 7 phosphate buffer and then spiked with known amounts of ibuprofen (0.25, 0.5, 1, 2, 5 and 10 ug) and 2.5 ug of p-methoxyphenylacetic acid as the internal standard.

2.9.2.3.0.0 STEREOSELECTIVE DISPOSITION OF TIAPROFENIC ACID IN ARTHRITIC SUBJECTS

2.9.2.3.1.0 DRUG ADMINISTRATION AND SAMPLE COLLECTION

Ten arthritic subjects participated in this study. Written consents were obtained following an explanation of the objectives and implications of the study. Four subjects were given racemic tiaprofenic acid orally, 200mg/8 hours (Surgam R capsules, Roussel) for at least one week. No other medication was allowed during the course of the study. On the day of the experiment, the first dose was given at least 1 hour before or after breakfast. Venous blood (4-5 mL) was drawn into heparinized tubes at 0, 0.25, 0.5, 1, 2, 3, 4, 6 and 8 hours and urine (total output) was collected at 0.5, 1, 3, 5, 7, 8 and 9 hours. To six subjects who required knee aspiration, single doses of 200 mg racemic tiaprofenic acid were given and synovial fluid was collected (using heparinized syringes) between 0.25-3 hours. Plasma (separated from blood), urine and synovial fluid were stored at -20°C until analyzed.

2.9.2.3.2.0 DETERMINATION OF EXTRACTION EFFICIENCY

Five blank plasma samples spiked with tiaprofenic acid (5 ug) were used to determine the extracion efficiency of toluene. A separate set of 5 toluene extracts of S-(+)-amphetamine were spiked with 5 ug of the drug (100 ug/mL in absolute ethanol). Both sets of samples were then derivatized and analyzed.

2.9.2.3.3.0 DETERMINATION OF THE EFFECT OF 1-HYDROXY-BENZOTRIAZOLE ON YIELDS OF TIAPROFENIC ACID-α-METHYLBENZENEETHAMINE DERIVATIVES

Five samples of toluene extracts containing the resolving agent and 5 ug of tiaprofenic acid were spiked with 10 ug of the triazole (100 ug/mL in toluene). The peak response of the diastereoisomers thus obtained was compared with that of similarly derivatized tiaprofenic acid samples which did not contain the triazole.

2.9.2.3.4.0 CALIBRATION CURVES FOR TIAPROFENIC ACID

Tiaprofenic acid concentrations in the biological samples were determined from standard curves obtained by spiking blank samples of plasma, urine or synovial fluid with known amounts of racemic tiaprofenic acid. The concentrations thus obtained were 0.25, 0.5, 1, 2, 5, 10 and 20 ug/mL of racemic tiaprofenic acid. The internal standard used was 5 ug of S-(+)-naproxen (25 uL of test solution).

2.9.2.4.0.0 STEREOSELECTIVE DISPOSITION OF ETODOLIC ACID IN A NORMAL AND AN ARTHRITIC SUBJECT

2.9.2.4.1.0 DRUG ADMINISTRATION AND SAMPLE COLLECTION

Subjects were a 53 year old female arthritis patient (subject F) who was under treatment with 200 mg/12 hours etodolac (Ultradol capsules, Ayerst) for over one month and a healthy 39 year old male (subject M). They received no other drugs. On the day of the experiment, subject F took her first daily dose of 200 mg and subject M his single 200 mg dose of etodolac. Blood (4-5 mL) and urine (total output) samples were collected at 0, 0.5, 1.5, 2.5, 3.5, 4.5, 6, 7, 8, 12 and 24 hours and 0, 1, 2, 3, 4, 5, 6, 7, 8, 12 and 24 hours respectively. Heparinized collecting tubes (grey top Vacutainers) were used to draw blood samples from the left arm vein. After the separation of plasma, specimens were kept frozen until the day of analysis.

2.9.2.4.2.0 CALIBRATION CURVES FOR ETODOLAC

For the purposes of calibration, 0.5 mL samples of blank plasma or urine diluted with 0.5 mL of pH 7 phosphate buffer were spiked with various aliquots of standard test solutions of etodolac to give a concentration range of 0.25, 0.5, 1, 2, 5, 10 and 20 ug/mL. The internal standard was S-naproxen (5 ug in 25 uL).

2.9.2.4.3.0 HPLC ANALYSIS OF ETODOLAC URINE CONJUGATES

Urine samples (0.5 mL aliquots in triplicate) obtained from subjects were mixed with 50 uL or 100 uL NaOH (1M) and acetonitrile (0.5 mL). Samples were then vortexed, centrifuged (3,000 rpm, 10 min) and filtered through 2 um disposable tips (Supelco). The filtrates were collected in 250 uL vials and a 100 uL aliquot of the sample was chromatographed using methanol:water:acetic acid (50:48:2) as the mobile phase; analytical wavelength, 280 nm.

The experiment was repeated on another aliquot of urine but without the addition of NaOH.

2.9.2.4.4.0 ISOLATION OF 7-HYDROXYETODOLAC

Pooled urine samples were hydrolyzed with NaOH (1M), extracted with ethyl acetate and the solvent removed under vacuum. The crude extract was purified by silica gel column chromatography using chloroform:ethanol (9.8:1.2 v/v). Forty fractions (30 mL) were collected and each fraction was analyzed. The 7-hydroxy metabolite was isolated from fractions 4-6 and analyzed by proton NMR, GC/MS and direct inlet probe MS.

For the purposes of GC analysis, the isolated metabolite or the urine samples were treated in a manner similar to that described in Section 2.9.2.1.0.0.. The dry residue of the diastereoisomers thus obtained was treated with trifluoroacetic anhydride (100 uL) and the reaction allowed to proceed for 1 minute. After evaporation of the reagent, the trifluoroacetylated residue was reconstituted in toluene (100 uL) and 1 uL used for GC analysis.

3.0.0.0.0.0 RESULTS AND DISCUSSION

3.1.0.0.0.0 MICROBIAL S-OXIDATION OF β-LACTAM DERIVATIVES

The chemistry of β-lactam-based antibacterial agents continues to be an area of intense development. Researchers in pharmacokinetics and pharmacodynamics have kept pace with the newly developed agents (e.g. the pharmacokinetics of third generation β-lactams has recently been reviewed (Ballant et al., 1985) and literature on the mode of action of β-lactam antibiotics has been updated (Tipper, 1985). Ironically, however, the potential of β-lactam sulfoxides as antibiotics has historically been overlooked most probably because of (1) the difficulties involved in the stereospecific chemical synthesis of sulfoxide enantiomers, and (2) the generally accepted notion that oxidation of sulfur is a metabolic deactivation process. As a consequence, the isomeric sulfoxides of β-lactams came to be regarded as inactive metabolites although several reports suggested otherwise (Cooper et al., 1969, 1970; de Konig et al., 1977; Durekheimer et al., 1977; Matula, 1978; Debono and Gordee, 1982; Dunn, 1985). It is interesting to note that as a metabolic process, sulfoxidation is second only to β-lactamase-induced hydrolytic cleavage in the β-lactam antibiotics (Flynn, 1977; Gottstein, et al., 1978).

In view of the potential stereochemistry-dependent antibacterial properties of sulfoxides (de Konig et al., 1977) it was desirable to utilize microorganisms for their preparation, particularly as an alternative to stereospecific chemical synthesis. Microbially-mediated synthesis was especially attractive by virtue of the mild reaction conditions, the inherent stereoselectivity of microbial enzymes, and the potential for scale-up to provide preparative quantities of the desired product (Fig. 14).

On the basis of earlier studies in these laboratories (Nazarali et al., 1982) the oxidizing potential of Cunninghamella echinulata was well known. However, it was necessary to conduct a preliminary, qualitative and comparative study in order to determine the most appropriate fungal genus for oxidizing a sulfur atom. Based upon HPLC analysis of several fungal incubates (see Section 2.7.1.2.2.0, Materials and Methods) it was determined that only C. echinulata, T. viride, and T. polysporum were capable of producing the sulfoxide metabolite. Comparison of peak heights, on the HPLC chromatograms, indicated that C. echinulata was more productive than the other two fungi.

The HPLC properties (Fig. 15) of the metabolite, compared favorably with those of authentic R-sulfoxide. However, since a non-stereospecific HPLC assay was utilized, the stereochemistry at the sulfur atom was not known; for C. echinulata incubates this was determined in a scale-up study. After purification, the metabolite was subjected to ^{1}H-NMR analysis. On comparison of the NMR spectrum with that of an authentic standard it was revealed that the metabolite had the R-configuration at sulfur (Table 9). This configurational assignment was further confirmed by comparison of the spectral data with those of structurally similar cephalosporin sulfoxides (Durekheimer et al., 1977).

Since the goal of the preparative study was to obtain sufficient metabolite for structural elucidation, no effort was made to maximize the yield. However, on the basis of the kinetic study (vide infra) the substrate concentration should not exceed the saturation limit of 100 ug/mL. Although a sulfone product was not observed, the addition of acetone powder (Smith and Rosazza, 1975) would be advisable to prevent its possible formation and thereby maximize the sulfoxide yield.

Cultures added to Bacto Sabouraud broth resulted in excellent growth but production of the sulfoxide metabolite was inferior to that obtained with Czapek-Dox medium; this broth was subsequently used for all further studies.

In Figure 16 is depicted a typical HPLC chromatogram obtained from an extract of C. echinulata incubated with the methyl ester of 7-[(phenoxy)acetamido]desacetylcephalosporanic acid (CS) as the substrate. As expected with a reverse phase HPLC system the most polar component, the sulfoxide metabolite of CS (CSO), was first to elute (Rt 1.8 min) followed by CS (3.3 min) and internal standard, (the corresponding cephalosporanic acid ethyl ester), 7.5 min. The peak in the chromatogram corresponding to the metabolite CSO was absent in substrate control cultures (medium plus substrate but without microorganism). Similarly, the peaks corresponding to CS and CSO were absent in the analytes of medium control flasks (medium plus microorganism but without substrate).

Concentration-time curves for CS and CSO are shown in Figures 17A-20B. In Table 8 is listed the half-lives and rate constants for the disappearance of the substrate, CS, and the metabolite CSO. The kinetic parameters were obtained using the mean of 3 values. For quantitative analyses, calibration curves were obtained using concentrations of 5, 10, 20, 40, 80, 100, 200, 400 and 600 ug/mL of medium. The curves were linear ($r^2=0.992$) with excellent reproducibility (%CV<8, n=3).

As all the manipulations, i.e. inoculation, incubation and growth of microorganism, had been standardized, any interflask variation would be minimal. It was also assumed that under standard conditions, each culture flask was an independent entity analogous to the one compartment model used to describe mammalian pharmacokinetics. Using this analogy the effect of substrate concentration on the production of the metabolite could be observed. Normally the rate of disappearance of CS, in a fixed volume of _C. echinulata_ culture would be expected to obey first-order kinetics. This is clearly evident at CS concentration levels of 25 and 100 ug/mL of medium (Figs. 17A and 18A). However at concentrations of 200 and 400 ug/mL (Figs. 19A and 20A) an accumulation of substrate in the medium is observed. One explanation is saturation of the CS transport mechanism and consequently poor uptake. This would imply zero-order uptake and therefore concentrations greater than 200 ug/mL are beyond the saturation limits for microbial uptake of CS.

The values obtained for Vmax and Kmax (Table 8) further support the occurrence of first-order uptake kinetics at lower concentrations and zero-order at higher concentrations. The observed increase in the rate of metabolism of CS to CSO with increasing substrate concentration (and with the capacity, Kmax, remaining unchanged) may be due to transient enzyme inductive properties of the cephalosporins, a phenomenon comparable to the cephalosporin-induced transient elevation of hepatic enzymes in humans (Balant et al., 1985).

On comparison with mammals, it would be reasonable to assume that in the microbial system metabolism plays an important role in drug elimination. The observed rate formation constant (KF) values for CSO (at CS levels of 25 and 100 ug/mL) (Table 8) are indicative of either (1) concentration-dependent kinetics, or (2) time-dependent kinetics. Due to the limited number of data points, the values could not be determined for the 200 and 400 ug/mL concentrations. However, based on the disappearance rate constant (KE) for CS, it would be reasonable to assume that the metabolism of CS to CSO is concentration-independent.

It is possible that in this microbial system disappearance of the sulfoxide metabolite is observed due to subsequent oxidation to the sulfone or as a result of other metabolic pathways such as C-S bond cleavage (Hanzlik and Cashman, 1983) and β-lactam ring-opening. The disappearance rate constant values for CSO, with various levels of CS, are indicative of a dose-dependent phenomenon. Since KF is greater than KE, the rate of further metabolism of CSO to CSO_2, or undetermined products, is slower than its formation. These observations are in agreement with the fact that microbial oxidation of sulfur proceeds in a step-wise manner (Smith and Rosazza, 1975). Consequently, the zero-order kinetics of oxidation of sulfoxide to sulfone should lead to the accumulation of CSO at higher concentrations of CS. This would explain the longer half-lives of CSO at CS levels of 200 and 400 ug/mL.

Based on the above arguments the kinetic model depicted in Figure 21 is proposed and the following conclusions are presented: (1) the rate of CS disappearance is a capacity-limited process, exhibiting first-order kinetics at lower concentrations and zero-order at higher concentrations. This is also the rate-limiting step in the oxidative metabolic process; (2) the oxidation of CS to CSO is dose-independent and therefore obeys linear kinetics; (3) the metabolic oxidation or degradation of CSO again follows zero-order (non-linear) kinetics, leading to CSO accumulation at higher levels of CS.

During the course of this study it was anticipated that aromatic hydroxylation might occur since the substrate CS has a phenoxy group as part of the C7 substituent. Although such metabolite(s) were not observed, their formation could not be precluded. The mechanisms for sulfoxidation and aromatic hydroxylation by microsomal enzyme systems differ at cellular levels. A study undertaken by other investigators (Cashman and Hanzlik, 1981, 1983) is relevant to the present discussion. The study involved the hepatotoxin thiobenzamide (Fig. 22) which is extensively oxidized by microsomal enzymes to the corresponding sulfoxide, even though it is also a potential target for aromatic hydroxylation. The sulfur mono-oxygenated product was the major metabolite formed initially. When thiobenzamide was incubated along with biphenyl, as a standard for comparison, two discriminative patterns were observed. Cytochrome P-450 involvement (NADPH dependence and inhibition by N-octylimidazole, SKF-525A and secondary cyclopropylamines) occurred with biphenyl, thiobenzamide was metabolized by microsomal FAD-containing mono-oxygenases responsible for sulfoxidation. It appeared that not only were there two different enzymes involved in the metabolic oxidation of sulfur and aromatic carbon, but also that the microsomal FAD-containing mono-oxygenases may be the dominating entity.

As the initial aim of this thesis research was to determine and demonstrate the metabolic capability of microorganisms to oxidize sulfur, and subsequently deduce the stereochemistry of the product, the above-mentioned enzymatic aspects in C. echinulata metabolism were not investigated further.

3.2.0.0.0.0 MICROBIAL p-HYDROXYLATION OF β-ADRENOCEPTOR DRUGS

(+/-)-Prenalterol has been analyzed in biological samples using HPLC (Oddie et al., 1982; Lagerstrom and Carlebom, 1984) and electron-capture GC (Degen and Ervik, 1981). The method utilized in this study was similar to a reported GC-MS procedure (Ervik et al., 1982) except that trifluoroacetic anhydride was used for derivatization followed by nitrogen-phosporus detection. A typical chromatogram is shown in Figure 23. The retention times of TFA-derivatized (+/-)-pre-prenalterol, (+/-)-prenalterol, and internal standard were 5.09 min, 6.14 min and 8.34 min respectively. On GC-MS comparison with similarly derivatized authentic standards, the components were identified as N,O-bis(trifluoroacetyl)pre-prenalterol, N,O,O,-tris(trifluoroacetyl)prenalterol, and N,O-bis(trifluoroacetyl)- propranolol (Figs. 24, 25A-C). Typical calibration curves could be described by $y = 0.715x - 0.004$ for (+/-)-pre-prenalterol ($r^2 = 0.997$) and $y = 0.421x - 0.049$ for (+/-)-prenalterol ($r^2 = 0.984$).

The concentration-time curves (n=5) of pre-prenalterol and prenalterol for three substrate concentrations are depicted in Figures 26-28. The kinetics of pre-prenalterol formation by C. echinulata were typically linear at low concentrations but non-linear at higher concentrations. With initial concentrations of 100 and 200 ug/mL, the disappearance of (+/-)-pre-prenalterol from the broth proceeds according to first-order kinetics. As expected, the plot of CRF (concentration of prenalterol remaining to be formed) was log-linear and superimposable with the concentration-time plot of the substrate (Figs. 26, 27). This indicates that the biotransformation of (+/-)-pre-prenalterol is mainly, if not exclusively, through the formation of (+/-)-prenalterol (Fig. 29). The preparative yields of greater than 85% (Table 10) further support this suggestion. However, as shown in Figure 28, with a (+/-)-pre-prenalterol concentration of 400 ug/mL, the kinetic pattern changes considerably. The CRF plot had two distinct phases; an initial slow rate of (+/-)-prenalterol formation followed by a log-linear phase, with a slope similar to that estimated from the lower concentration curves (Table 10). Thus with substrate concentrations greater than 200 ug/mL, the kinetics of (+/-)-prenalterol formation and (+/-)-pre-prenalterol uptake appear to be non-linear with Km of 427.811 ug/mL and Vmax of 232.301 ug/mL.day. This suggests that the C. echinulata- mediated para-hydroxylation of (+/-)-pre-prenalterol to (+/-)-prenalterol proceeds through a single capacity-limited pathway (vanGinnekan et al., 1974). The initial substrate concentrations of 100 and 200 ug/mL were lower than the observed Vmax and therefore, in these instances, the biotransformation proceeds with apparent first-order kinetics.

The limited capacity of the hydroxylating enzymes of C. echinulata is also evident in the (+/-)-pre-prenalterol 400 ug/mL concentration-time plot (Fig. 28). The initial slower disappearance of the substrate was preceded by a rapid phase indicating very quick uptake by the microorganism. Further evidence for rapid uptake was obtained when the incubates were examined following addition of substrate; after 24 h of incubation the media:cell distribution ratio was found to be 0.483(mean) +/- 0.082(sd).

From the preceding discussion it is apparent that for a first-order biosynthesis of (+/-)-prenalterol, the initial substrate concentration must be lower than the metabolic capacity of the microorganism. However, even at a substrate concentration of 400 ug/mL there is virtually complete conversion to (+/-)-prenalterol after 8 days of incubation (Table 10). Extraction of the mycelial cake followed by GC analysis of the extract revealed that only trace amounts of prenalterol remained within the microbial cells.

The product obtained from the preparative fermentation was confirmed to be prenalterol by comparison with the authentic synthetic standard. The melting points, ^{1}H-NMR (Table 11), IR and mass spectra were identical.

3.2.1.0.0.0 MICROBIAL VS CHEMICAL SYNTHESIS OF RACEMIC PRENALTEROL

Previous studies with β-adrenoceptor antagonists suggested that C. echinulata was a useful model of mammalian metabolism, specifically, p-hydroxylation of a phenyl ring (Nazarali et al., 1982). It was anticipated, therefore, that pre-prenalterol would similarly undergo hydroxylation at the position para- to the oxypropanolamine side chain. (+/-)-Pre-prenalterol was easily prepared from phenol, in two synthetic steps, using relatively inexpensive reagents. Regiospecific microbial hydroxylation completed the synthesis. The same reagents (epichlorhydrin and isopropylamine) were utilized for a total chemical synthesis of (+/-)-prenalterol. In this case, however, four synthetic steps are required if p-benzoquinone is the starting material. One phenolic function must be initially protected in order to construct the oxypropanolamine chain on the opposite side of the ring. The last deprotecting step affords (+/-)-prenalterol.

Although hydroxylation of the aromatic ring is regiospecific, this metabolic route is also non-stereoselective, i.e. the microorganism metabolizes (+)- and (-)-pre-prenalterol to the same extent. The high yields (Table 11), absence of any substrate after 8 days of incubation, and the formation of a single metabolite, substantiate the non-stereoselective nature of C. echinulata-mediated aromatic hydroxylation of racemic pre-prenalterol. Utilization of (-)-pre-prenalterol as the substrate should give (-)-prenalterol (the pharmacologically active isomer) and consequently a potentially attractive alternative to exclusive chemical synthesis.

If mammalian dose-dependent pharmacokinetics, with an emphasis on Phase 1 oxidative biotransformations (Dayten and Sanders, 1983), are extrapolated to the microbial system, then the limited metabolic capacity of C. echinulata may be attributed to either (1) overloading of the metabolizing system; or (2) enzyme inhibition by substrate or metabolite; or (3) a combination of factors (1) and (2).

Experimentally it is difficult to confirm the singular possibilities (1) or (2). Enzyme saturation and, to some extent, substrate inhibition may be factors jointly responsible for the limited metabolic capacity of the microorganism. The mammalian oxidative systems, which involve active forms of oxygen, are known to be inhibited under conditions of substrate overload (Payne, 1978). Since aromatic hydroxylations proceed via intermediate arene oxides (Nelson and Powell, 1979) it is plausible that the microbial oxidative system is similarly inhibited. The coexistence of an alternate mechanism, such as enzyme inhibition by the substrate, may be of some significance since pretreatment of rat microsomal fractions with propranolol is known to inhibit its metabolism (Pritchard et al., 1980).

An aspect that was not investigated was the effect of increased metabolic capacity at a constant volume of distribution. The data presented above suggest that limited metabolic capacity is the rate-limiting step in the bioproduction of prenalterol. An increase in the volume of the Phase II microbial inoculum should therefore result in enhanced metabolic capacity and hence linear kinetics for a corresponding higher substrate level.

3.3.0.0.0.0 GENERAL CONCLUSION FOR MICROBIAL METABOLISM STUDIES

The multi-faceted, kinetics-related, aspects of C. echinulata in the metabolism of CS and PP further illustrate the heterogeneity of microbial enzymatic systems. Substrate-associated differences in the kinetics of uptake and metabolism, substrate specificity of the metabolic enzyme systems, and stereo- and regiospecificity of these enzymes are significant features of microbial metabolism in general, and of C. echinulata in particular.

The potential of M^4 (microbial models of mammalian metabolism) systems and microbial catalysts in synthetic chemistry has received considerable attention (Smith and Rosazza, 1975; Perlman, 1979; Sebek and Perlman, 1979), but the feasibility of using these systems as "microbial models for mammalian pharmacokinetics" may be an innovative proposition. The potential and validity of such a proposition requires a thorough investigation. It should, however, be realized that relative to mammalian models, microorganisms are comparatively uncomplicated metabolic units. Many of the variables encountered during a mammalian study are not present in M^4 investigations. The simplicity of the microbial model might, however, be used to advantage for studying the kinetics of drug elimination via Phase I oxidative biotransformations. Such information, when extrapolated to mammalian systems, would be of assistance in the evaluation of the role of other metabolic pathways which contribute to drug clearance and elimination.

3.4.0.0.0.0 MAMMALIAN STEREOSELECTIVE DISPOSITION OF RACEMIC FENFLURAMINE

3.4.1.0.0.0 STEREOSPECIFIC ANALYSIS OF RACEMIC AMPHETAMINE ANALOGS

Racemic pre-prenalterol had been used as the substrate in the microbial biosynthesis of prenalterol. It was of interest to investigate whether microbial p-hydroxylation was a stereoselective metabolic process by determining the optical purity of the prenalterol product. Although not known at the time, subsequent polarimetric analysis of the prenalterol obtained from the preparative fermentation revealed that it was racemic.

Since the optical isomers of prenalterol were not readily available it was decided to obtain some familiarity with chromatographic chiral separation methods by using suitable model compounds. Amphetamine, and related amines, were selected because several optically pure isomers were immediately available. In addition, since prenalterol was also an amine, the chiral derivatizing agents utilized with the model compounds might be applied to the analysis of this drug.

Amphetamine, and closely related analogs (Fig. 30), were resolved as diastereoisomeric derivatives of

N-trifluoroacetyl-L-prolyl chloride. The separation factors (α) and retention times (Rt) of these derivatives are listed in Table 12 and their structures are shown in Figure 31. All N-TPC diastereoisomers were resolved to baseline on the fused silica capillary column (Figs. 32, 33). It is interesting to note that although the fluoroamphetamine and methamphetamine diastereoisomers were eluted later than the amphetamine derivatives, they were not as well resolved as the amphetamine isomers. This observation suggests a superior capacity factor, but lower separation factor, and implies that higher capacity factors do not necessarily mean better resolution of diastereoisomers. On the basis of the three point rule (Dalgliesh, 1952), the superior separation of (R)-AM-TPC and (S)-AM-TPC derivatives could be attributed to hydrogen bonding interactions between stationary phase and the amide group. Non-bonding interactions (stearic hindrance) would also occur as a result of the proximal proline group. These comments are in agreement with the observations of Karger-Westley-Halpern (Karger et al., 1967; Westley et al., 1968). After derivatization with N-TPC, methylamphetamine no longer has an amide proton available for hydrogen bonding with the stationary phase and resolution is consequently diminished slightly. Resolution is still observed because non-bonding interactions are still possible as well as hydrophobic interactions between the aromatic ring (probably pi-pi interactions; Pirkle, 1981) and the stationary phase.

It is apparent that while the amide proton does not appear to be exclusive requirement for separation, its presence does have a beneficial effect. Although the aromatic hydrophobic interaction provides one of the three interaction points, compared to the amide proton the ring is relatively further away from the chiral centre. The hydrophobic interaction should consequently make less of a contribution to the process of chiral recognition (Rose et al., 1966). The importance of the amide proton in chiral discrimination is further evident with fluoroamphetamine-TPC diastereoisomers. The high electronegativity of fluorine is known to hinder separation (Watabe and Gil-Av, 1985). It would therefore be expected that (+)- and (-)-fluoroamphetamine-TPC diastereoisomers would not be as well resolved as AM-TPC derivatives. However, the detrimental effect of fluorine is partly offset by the hydrogen bonding amide proton and consequently the resolution is better than that observed with methamphetamine-TPC diastereoisomers.

The superior separation of p-chloro-fluoroamphetamine diastereoisomers may be attributed to greater steric hindrance (non-bonding interactions) and that of tranylcypromine to overall conformational rigidity of the structure (see Section 1.2.1.1.1.1.a, Introduction).

In evaluating the performance of a fused silica capillary column (cross-linked dimethylsilicone) and a packed OV-101 (dimethylsilicone) column (Table 13) it is apparent that judgements made solely on the basis of separation factors may be misleading. Although the α values obtained on the packed column are superior to those on the capillary column, the chromatographic peaks are not resolved to baseline; baseline resolution is a desirable feature for quantitative analyses. This observation suggests that α values alone should not be used to compare the chiro-optical resolving power of capillary vs packed columns, even though they may incorporate the same stationary phase. The superior HETP value as well as greater length enables the capillary column to effect baseline separations despite lower α values. Thus the native stereochemical properties of the diastereoisomers are not solely responsible for peak resolution; selection of an appropriate GC column and operating conditions are also important. A typical GC resolution on a packed column, of methylamphetamine-N-TPC diastereoisomers is depicted in Figure 34.

This concept is illustrated further with the example following. Even though the diastereoisomeric derivatives of amphetamine obtained with (-)-camphanic acid chloride were readily formed (MS data, Figs. 35 and 36), they were not separable (Fig. 37) under the conditions described in Section 2.8.2.0.0.0, Materials and Methods. This was particularly surprising since camphanic acid is a highly rigid molecule, a desirable characteristic for a chiral derivatizing agent. Successful separations (e.g. proxyphylline by reverse phase HPLC) have been reported with this reagent (Ruud-Christensen and Salvesen, 1984). Failure to resolve the camphanic acid amide derivatives of amphetamine may be attributed to at least three factors: (1) intra-molecular interactions (hydrogen-bonding?) between the amide and lactone groups resulting in an overall loss of sites for stereochemically controlled interations between the diastereoisomer and the stationary phase; (2) non-discriminative dipole moments of the derivatives. As described for ibuprofen ester derivatives (Wainer and Doyle, 1984), if the differences between the dipole moments of the diastereoisomeric camphanic acid amides are relatively insignificant, then chiral separation will be difficult to achieve; (3) the chromatographic conditions (e.g. GC column, operating parameters) were not conducive to separation.

3.4.2.0.0.0 RELATIONSHIP BETWEEN ORDER OF ELUTION AND ABSOLUTE CONFIGURATION/OPTICAL ACTIVITY OF RESOLVED AMPHETAMINE ANALOGS

The order of elution of N-TPC derivatized amphetamine (Fig. 38) and methamphetamine was correlated with the absolute configuration. In both cases the R-(-)-isomers eluted first. This is in agreement with other studies conducted on N-TPC derivatives of amphetamine and methamphetamine (Liu and Ku, 1981, 1983; Liu et al., 1982). With fluoroamphetamine, the (-)-isomer is again the first to elute (Fig. 39). If, as it has been suggested, there is a correlation between order of elution and absolute configuration (Gil-Av and Nurok, 1970, 1974; Schurig, 1984), then the (-)-fluoroamphetamine isomer should also have the R-configuration. However, due to the higher priority of fluorine, application of the Cahn-Ingold-Prelog sequence rules (Bassindale, 1984) would reverse the configurational assignment and therefore the (-)-fluoroamphetamine isomer would have the S-configuration. The same argument applies to p-chloro-fluoroamphetamine.

Due to the unavailability of optically pure isomers of tranylcypromine and fluorotranylcypromine, a correlation between order of elution and absolute configuration could not be established. However, it has been suggested that in similar diastereoisomers, the order of elution is determined by the absolute configuration at the asymmetric benzylic carbon (Gal, 1984). It is proposed that the first eluting peak of derivatized tranylcypromine corresponds to the 1R, 2S-isomer which is levorotatory (Riley and Bryer, 1972). This is in agreement with N-TPC-derivatized amphetamine and methylamphetamine in which the first-eluting isomers have the R-(-)-configuration. A similar association between amphetamine and tranylcypromine was also observed when R-(-)- or S-(+)-benoxaprofen chloride were used as chiral derivatizing reagents (Weber et al., 1984).

The electron impact-mass spectral fragmentation patterns of the N-TPC derivatives of amphetamine, fluoroamphetamine and methamphetamine are shown in Figures 40-43. A number of similarities are evident. Molecular ions were not observed but all diastereoisomers gave the N-(trifluoroacetyl)- pyrroline fragment (m/z 194-CO) arising from the prolyl portion of the derivative. Another characteristic fragment from the same region of the molecule was m/z 194. The three amines can, however, be easily distinguished due to the fragments obtained after the loss of the benzyl radical. These fragments were m/z 237, 255 and 251 from the amphetamine, fluoroamphetamine and methamphetamine diastereoisomers respectively. In the mass spectra of derivatized fluoroamphetamine (Figs. 40, 43) the m/z 255 fragment loses a molecule of hydrogen fluoride to give a fragment of low abundance, m/z 235 (5%).

3.4.3.0.0.0 **MAMMALIAN N-DEALKYLATION OF FENFLURAMINE**

A simultaneous separation of the diastereoisomeric N-trifluoroacetyl-L-prolyl amides of amphetamine, methamphetamine and fluoroamphetamine was possible (Fig. 33), suggesting that this analytical method would be useful in metabolic studies. It was decided to verify the applicability of the analytical method to a study of the stereoselective disposition of a racemic, clinically relevant amine drug in a biological model. The anorectic agent fenfluramine was selected because of its structural similarity to the model compounds. This drug is preferentially metabolized to pharmacologically active norfenfluramine (Beckett and Brooks, 1967) which would be simultaneously resolved as an N-TPC derivative. The identities of fenfluramine and norfenfluramine enantiomers (as N-TPC diastereoisomers) in the biological samples were confirmed by GC-MS (Figs. 49-51).

The calibration curves of the respective isomers of fenfluramine and norfenfluramine were superimposable. In

Figure 44 is depicted a typical gas chromatogram obtained after extraction and derivatization of the drug and its metabolite from rat brain; the internal standard was (+)-fluoroamphetamine. The concentration-time curves of accumulated fenfluramine and norfenfluramine isomers in rat brain tissue are shown in Figures 45 and 46 respectively. Similarly, the concentration-time curves of fenfluramine and norfenfluramine isomers in blood-free liver tissue samples are shown in Figures 47 and 48 respectively. The levels of fenfluramine and norfenfluramine enantiomers in liver tissue were found to be consistent with the levels in brain.

The results obtained confirm and extend previous observations that the R-(-)-isomer of fenfluramine is preferentially N-dealkylated to norfenfluramine (Beckett and Brooks, 1970; Morgan et al., 1972), particularly in the rat (Jori et al., 1978; Caccia et al., 1981, 1982). Consequently, stereoselective hepatic extraction of the R-isomer of fenfluramine would explain the observed higher levels of S-isomer, and conversely the higher levels of R-norfenfluramine. Differences in the levels of fenfluramine are basically due to metabolic stereoselectivity since the absorption of fenfluramine from the peritoneal cavity is non-stereoselective (Jori et al., 1978). It is evident from the concentration-time plots (Figs. 45 and 46) that the brain acts as a storage site for both fenfluramine and norfenfluramine. A constant difference in the levels of R- and S-fenfluramine (paired T test p.and norfenfluramine (paired T test $p<0.001$), for the time period under consideration, is indicative of enzyme saturation or, more precisely, a capacity-limited N-deethylation process. In a separate, but similar, study (Caccia et al., 1982) it was demonstrated that although the kinetic parameters of fenfluramine isomers in the rat may differ markedly with higher (saturation) doses, the disposition kinetics of the norfenfluramine isomers were superimposable. This evidence suggests enzyme saturation and subsequent loss of stereoselectivity. It may also be inferred that the major metabolic pathway of fenfluramine (isomers) is indeed by N-deethylation and that saturation of this pathway results in accumulation of the drug. The rate of disappearance of both fenfluramine isomers is dose-dependent resulting in a significant effect on the disposition of the R-(-)-enantiomer.

It [illegible]d not be concluded that the pharmacological stereoselectivity results from differences in the pharmacokinetics of the enantiomers. Although anorectic activity is mainly due to the S-(+)- enantiomer in humans, the overall pharmacokinetics of the enantiomers are virtually identical (Caccia et al., 1982).

The studies described above provided significant expertise in the developement of stereospecific assays and their application to the study of stereoselectivity in drug disposition. It was appropriate at this time to apply the expertise to a therapeutic problem. The arylalkanoic class of non-steroidal antiinflammatory drugs was selected, in part, because of the widespread use of these agents but more importantly because of the lack of correlation between therapeutic efficacy and drug blood levels. The amphetamine enantiomers studied previously were utilized as chiral resolving agents in the stereospecific analysis of racemic arylalkanoic NSAIDs.

3.5.0.0.0.0. MAMMALIAN CHIRAL INVERSION AND DISPOSITION OF 2-ARYLALKANOIC NSAIDs

The stereoselective disposition (absorption, distribution, excretion) of chiral 2-arylpropionic acid non-steroidal antiinflammatory drugs (Fig. 52) has recently attracted significant attention (Hutt and Caldwell, 1983, 1984). Of particular interest is the unidirectional bioconversion of the distomer (R-configuration, generally levorotatory) to the eutomer (S-configuration, dextrorotatory). It becomes apparent that a derivatization procedure for the resolution and measurement of the individual enantiomers present in biological fluids was essential for the proper interpretation of pharmacokinetic data obtained following the administration of the drugs as racemates.

The quantification of 2-APA enantiomers in biological media has been reported using HPLC, GC, TLC and radiochemical methods (Hutt and Caldwell, 1983). Of these, HPLC and GC appear to be the most frequently applied techniques; with a few exceptions (Goto et al., 1982; Lee et al., 1985), optically active α-methylbenzylamine (MBA) has been the most popular derivatizing reagent for both chromatographic procedures. Thus, diastereoisomeric NSAID-α-methylbenzylamides are prepared directly by means of a coupling reagent such as CDI (Bopp et al., 1979; Kemmerr et al., 1979; Stoltenberg, 1981; Maitre et al., 1984; Rubin et al., 1985), or via the NSAID acid chloride prepared with thionyl chloride (Tossolini et al., 1972; McKay et al., 1979; Simmonds et al., 1980; Tamassia et al., 1984).

During the course of this research, it was determined that the thionyl chloride procedure was satisfactory for the preparation of diastereoisomeric ibuprofen-methylbenzeneethanamides (Fig. 53) and fenoprofen-methylbenzene- ethanamides. However, this route was found to be ineffective for the preparation of diastereoisomeric tiaprofenic acid-benzeneethanamides. On the basis of proton NMR evidence it appeared that reaction with thionyl chloride resulted in degradation of the thiophene ring present in tiaprofenic acid. In addition, it has been reported (Kaiser et al., 1976) that the hydroxylated metabolites of ibuprofen are readily dehydrated and chlorinated with thionyl chloride and the halogenated product subsequently dehydrochlorinated. Since a general method, equally applicable to the analysis of metabolites, was required for the purposes of this thesis, the thionyl chloride route was not pursued.

There are comparatively few reports describing GC methods for the separation of NSAID enantiomers present in biological media. Racemic ibuprofen (Brooks and Gilbert, 1974; Vangiessen and Kaiser, 1975; Kaiser et al., 1976; Cox et al., 1985) and benoxaprofen (Bopp et al., 1979) have been resolved as their MBA derivatives using GC with flame ionization detection. When MBA is used as a derivatizing reagent, a large excess (6.5 mg) of CDI is required for the analysis of ibuprofen (0-50 ug) (Vangiessen and Kaiser, 1975). It has recently been reported (Maitre et al., 1984) that increasing the proportion of CDI results in a corresponding increase in derivative yield. In that study, 52 mg of CDI were used for the derivatization of several NSAID (10-140 ug) with MBA. Formation of the symmetrically disubstituted α-methylbenzyl derivatives of urea, by reaction of MBA with the excess CDI, is minimized by the addition of acetic acid.

Dicyclohexylcarbodiimide (DCC)-mediated couplings in polypeptide syntheses were found to be essentially quantitative when equimolar amounts of DCC were used (Windridge and Jorgensen, 1971). On this basis, it was felt that reduced quantities of chemically related CDI would be effective and consequently only 10 ug of CDI were utilized in the derivatization of 10 ug of NSAID. Since CDI is not present in large excess, the resulting chromatograms are virtually free of any interfering peaks (Figs. 54-57). When DCC was used as a condensing agent, numerous spurious chromatographic peaks were observed and therefore this reagent was unsuitable.

As an alternative to MBA, optically pure amphetamines were utilized for the resolution of arylalkanoic NSAIDs. The reaction steps involved in acylation with thionyl chloride and CDI are depicted in Figures 58 and 59. Using the CDI method, calibration curves for ibuprofen were linear (coefficient of correlation, 0.996-0.999) over the concentration range of 0.25-10 ug/ml of plasma or urine. The minimum quantifiable concentration was 75 ng/ml of biological sample, with an on-column detection limit of 0.75 ng. A typical calibration curve could be described by $y=0.024 + 0.185x$ for the S-isomer and $y=0.029 + 0.155x$ for the R-isomer. When the quantity of CDI was increased to 50 ug, a significant amount of an unidentified component was obtained (Fig. 60). This substance is apparently formed via reaction between AM and CDI as it also appears in the reagent blank sample where an NSAID is not present. According to mass spectral evidence, it was not the α-methylphenylethyl derivative of urea. For the purposes of comparison, when ibuprofen (Figs. 61, 62) and fenoprofen (Figs. 63, 64) were derivatized with MBA, using the recommended (Vangiessen and Kaiser, 1975) quantities of CDI, numerous prominent peaks appeared in the chromatographic traces, particularly in the region prior to elution of the diastereoisomers. These peaks were observed, in part, as a result of the greater resolving capability of capillary compared over packed columns (used in earlier investigations). In addition, because of the column operating conditions (Section 2.9.1.0.0.0.b, Materials and Methods), several volatile components are detected which would normally co-elute with the solvent when higher oven temperatures are used. It was felt that under these chromatographic conditions, MBA produced too many potentially interfering peaks in an area of the GC trace where metabolites might occur and, in addition, reduced the lifetime of the column. When the quantity of CDI was reduced to 10 ug, no reaction was observed between MBA and the NSAID.

A further comparison of MBA with AM revealed that AM consistently gave a chromatographic peak area ratio (i.e. ratio of peak areas of the two diastereoisomers) of 0.90-1.0 for all the NSAID diastereoisomers in this study. MBA gave equally good results with the two NSAIDs, fenoprofen and ibuprofen, selected for comparison. A ratio value of 1.0 is, of course, ideal in the analysis of racemates and suggests that the derivatizing reagent reacts equally well with both enantiomers. When the value deviates from unity, the calculation of enantiomer ratios in test samples requires the incorporation of a predetermined correction factor.

AMP and MBA were essentially comparable in providing baseline resolution of the diastereoisomers, although peak separation was slightly greater with MBA. The retention times are also comparable; for example, ibuprofen-MBA diastereoisomers elute in approximately 15 min and the corresponding AMP derivatives in 17 min using the same chromatographic conditions.

MBA is commercially available and conveniently handled as a liquid. In these laboratories AMP free base is regenerated from the sulfate salt, thereby introducing an additional extraction step. However, the sulfate salts are stable and easily recrystallized if desired. Furthermore, it was observed that during storage at 4°C, a colorless deposit formed in the neck of the bottle containing MBA. The nature of this material was not determined nor was it clear whether this is a reflection of the stability of MBA.

It was essential to heat the reaction mixture as NSAID-AM derivatives were not formed at room temperature. Maximum yields (based on chromatographic peak areas of the diastereoisomers) were obtained after heating for 2 hours at 85°C. Increasing the heating time to 3 hours did not significantly affect the peak ratios or areas.

The identity of the chromatographic peaks listed in Table 14 is based on the following information: (1) optically pure S-(+)-ketoprofen was added to racemic ketoprofen and the mixture derivatized with S-(+)-AMP. The area of the peak with Rt=17.11 min was augmented (Fig. 65), indicating that the first-eluting stereoisomer has the S,S-configuration. When R-(-)-AMP was the resolving agent, the second-eluting peak was augmented, and therefore this diastereoisomer has the S,R-configuration; (2) naproxen is marketed as the S-(+)-enantiomer. When derivatized with S-(+)-AMP, a sample of naproxen gave two peaks (96:4 ratio), the major, and first-eluting, peak being the S,S-diastereo- isomer and the minor component, presumably the R,S-isomer. The order of elution reversed when R-(-)-AMP was used as derivatizing agent (Fig. 66). This information, along with similar results following HPLC analysis (adaptation of the method reported by Bjorkman, 1985 for the analysis of indoprofen), suggested that the minor component did, in fact, arise from the presence of a small amount of R-(-)-naproxen; (3) it has been shown that following the administration of racemic ibuprofen to healthy volunteers, the S-(+)-isomer predominates in both plasma (Vangiessen and Kaiser, 1975; Lee et al., 1985) and urine (Vangiessen and Kaiser, 1975). Following similar studies in these laboratories, the predominant isomer was found to elute first when derivatized with S-(+)-AMP and therefore this diastereoisomer was assigned the S,S-configuration; (4) it has been suggested (Gil-Av and Nurok, 1974; Schurig, 1984) that for closely related compounds the relationship between the order of elution and configuration is generally consistent. Thus, with several structurally similar diastereoisomeric amides synthesized from R-(+)-MBA, the S-acid,R-amine diastereoisomers eluted (HPLC) prior to the R-acid,R-amine isomers (Sonnet and Heath, 1982). The same elution order (HPLC) was observed in resolving racemic benoxaprofen with R-(+)-MBA (Simmonds et al., 1980). When the diastereoisomers are separated by GC methods, however, the elution order is reversed compared to HPLC, i.e. the S-acid,R-amine diastereoisomers of simple amides (Sonnet and Heath, 1982), as well as ibuprofenyl amides of optically pure MBA (Brooks and Gilbert, 1974), are retained longer. Since the order of elution of enantiomers reverses upon reversal of GC column phase chirality (Bonner and Blair, 1974; Schurig, 1984), it is anticipated that reversing the chirality of the resolving agent should similarly reverse the elution order of the corresponding diastereoisomers. Thus, when S-(-)-MBA was used to derivatize benoxaprofen (Bopp et al., 1979) and ibuprofen (Vangiessen and Kaiser, 1975; Kaiser et al., 1976), the first-eluted GC peak corresponds to the S,S-isomer. As expected, when analyzed by HPLC, the more polar S,S-isomers are retained longer on the normal phase columns (Simmonds et al., 1980; Stoltenborg et al., 1981; Goto et al., 1982; Maitre et al., 1984).

The elution orders reported in Table 14 are consistent with the preceding discussion; with S-(+)-AMP as resolving agent, the S-acid,S-AMP diastereoisomer elutes first while with R-(-)-AMP, the S-acid,R-AMP isomer is retained longer than the corresponding R-acid,R-AMP diastereoisomer.

The absolute configurations of (+)- and (-)-etodolic acid have not been reported. When reacted with S-(+)-AMP, the (+)-ET,S-AM and (-)-ET,S-AMP diastereoisomers had retention times of 17.08 and 18.75 min. respectively. Similar times were observed for the (-)-ET,R-AMP (17.04 min) and (+)-ET;R-AMP (18.69 min.) isomers. If the S-configuration-dextrorotatory relationship (observed with the 2-APAs) also holds for ET, then the first-eluting isomers should have the S,S- and R,R-configurations when S-(+)-AMP and R-(-)-AMP, respectively, are the reactants. However, it must be recognized that ET is not a 2-APA and, in this respect, is not closely related to the other NSAIDs studied in this investigation. An additional difference is observed when comparing the extent to which the isomers are resolved, which, in the case of ET, is particularly noteworthy (approximately 2 min; Fig. 67). This observation may be explained by the presence of the asymmetric centre as part of a six-membered cyclic structure whose inherent conformational rigidity and immobility is further reinforced by the aromatic indole group. This conformational feature is absent in the 2-APAs (Fig. 52). As mentioned previously, conformational immobility assists in the creation of an asymmetric environment in the region of the amide group, thereby enhancing non-equivalent accessibility of the ET,AMP-diastereoisomers for interactions with the stationary phase (Karger et al., 1967; Westley et al., 1968).

As with ET, the absolute configurations of the tiaprofenic acid enantiomers have not been reported. In addition, due to the higher priority of sulfur over oxygen [Cahn-Ingold-Prelog sequence rules for assigning R-, S-configuration (Bassingdale, 1984)], the 2-APA enantiomers (compounds I-VIII, Fig. 52) which have the R- and S-configurations would be assigned S- and R- respectively when describing tiaprofenic acid isomers. Due to the unavailability of TA enantiomers, the configurational identity of the diastereoisomers could not be determined. Reaction of racemic TA with S-(+)-AMP gave GC peaks at 16.72 and 17.19 min and with R-(-)-AMP at 16.80 and 17.25 min.

Structures of the diastereoisomeric amides were characterized by electron-impact and chemical ionization MS. The spectra confirmed the stability of NSAIDs at the temperatures required for reaction with optically pure amphetamines. Stability was particularly important for TA and pirprofen since the former undergoes some degradation at room temperature (Jamali et al., 1984) while the pyrroline group in the latter is readily oxidized to pyrrole (about 25% at room temperature; Edgar et al., 1982; Colussi et al., 1985). TA and pirprofen diastereoisomers gave quasi-molecular ions of 378 and 369/371 respectively. Quasi-molecular ions were also observed for all other NSAIDs derivatized in this study. The probable fragments of the diastereoisomeric amides obtained via electron-impact MS are shown in Table 15. With the exception of etodolic acid, which is not a 2-APA, the spectra of derivatized S- and R-ibuprofen (Fig. 68) are representative of the fragmentation patterns (Fig. 69) observed for the 2-APA diastereoisomers. Molecular ions were present in the EI mass spectra of derivatized ketoprofen, fenoprofen, naproxen, flurbiprofen and etodolic acid. The spectra contained a weak, but characteristic, [M-117]+ fragment which arises by expulsion of a C_9H_9 radical from the amphetamine portion of the molecular ion. An appropriate structure for the [M-117]+ fragment is shown in Figure 70. Interestingly a similar fragment, corresponding to m/z 206, has been reported from a CSP-LC/MS analysis (positive ion chemical ionization mode) of ibuprofen-benzylamide (Crowther et al., 1984). However, the investigators suggested that this fragment resulted from fragmentation and rearrangement to yield the ibuprofen fragment.

Other characteristic fragments included an [M-91]+ ion via the loss of a benzyl radical, and fragments of m/z 91 [$C_6H_5CH_2$+], 119 [$C_6H_5CH_2CH$+CH_3], 118 [C_6H_5CH=$CHCH_3$]+, and 117 [C_6H_5C+=$CHCH_3$] which are also derived from the amphetamine portion of the molecule.

The [M-162]+ fragment was prominent and, with the exception of ketoprofen and flurbiprofen, was the base peak in all spectra. This ion is the result of benzylic cleavage of the NSAID portion of the molecular ion. In the case of ibuprofen, the resulting ion (m/z 161) was [C_4H_9-C_6H_4-CH+CH_3]. The corresponding ions for the other NSAIDs were: 209 (ketoprofen), 185 (naproxen), 197 (fenoprofen), 199 (flurbiprofen), 206, 208 (pirprofen), 193 (cicloprofen) and 215 (tiaprofenic acid).

An abundant [M-161]+ fragment was also observed and arises from the molecular ion by McLafferty rearrangement and expulsion of $C_6H_5CH_2CH(CH_3)N$=C=O. All derivatized 2-APAs gave this fragment; in the case of ibuprofen, the resulting ion (m/z 162) was [C_4H_9-C_6H_4-CH_2CH_3]+.

Derivatized etodolic acid fragments in a different manner because of the influence of the oxygen-containing ring system(Fig. 81). Diagnostic fragments present were the molecular ion, m/z 404, [M-29]+, m/z 378, [M-204]+, m/z

228 and m/z 214 derived from the etodolic acid portion of the amide, and the ion, m/z 91 ($C_6H_5CH_2^+$), arising from the amphetamine portion.

The method just described was facile, sensitive, and readily applied to the separation and quantification of ibuprofen, TA and ET in biological samples (as discussed below).

3.5.1.0.0.0 STEREOSELECTIVE STUDIES ON IBUPROFEN

The GC and concentration-time curves for the analysis of plasma and urine samples obtained from a subject administered a single 600 mg oral dose of R,S-ibuprofen (Motrin and Apo-Ibuprofen ; see Section 2.9.2.2.1.0, Materials and Methods) are shown in Figures 71-74 respectively. As expected, the isomers undergo rapid gastro-intestinal absorption and peak plasma concentrations are achieved within 30 min of administration. Based on regression analysis, the terminal half-lives were 2.2 hours for the S-isomer and 2.1 hours for the R-isomer. From the urinary excretion rate curve it is apparent that the S-isomer is excreted to a greater extent.

The amphetamine derivatization procedure was utilized to study the stereoselective bioavailability of racemic ibuprofen (Motrin, 600 mg film-coated tablets; Table

16). Peak drug concentrations were achieved between 0.5 to 3 hours (Tmax 1.39(mean) +/- 0.78(sd)). In all of the subjects, the value for Tmax was similar for both isomers, with the exception of subject number 4. There were no significant differences in the mean peak concentration (Cmax) values for either isomer (paired t test, $p<0.05$). The difference in the AUCs of the S- and R-isomers demonstrates the stereoselectivity associated with the disposition of the isomers. Less than 5% of the drug was excreted in urine, with a higher degree of stereoselectivity than was observed in plasma (Table 17). This was not surprising as approximately 80% of the total dose of ibuprofen is eliminated by metabolism (Albert and Gernaat, 1984). The mean plasma ibuprofen concentration-time profiles obtained from the above subjects are depicted in Figure 76. These results agree with other studies which described the stereoselective disposition of ibuprofen (Lee et al., 1984).

The overall enantioselectivity in the disposition of ibuprofen isomers is primarily due to the unidirectional inversion of distomer to eutomer along with preferential elimination (via excretion and biotransformation) of the eutomer. Lee et al. (1985) determined that approximately 63% of the administered dose of R-(-)-ibuprofen is inverted to the S-(+)-enantiomer. Inversion may only be partly responsible for the overall predominance of the S-isomer in urine and the observed dextrorotatory nature of two of the three metabolites of ibuprofen. The process of chiral inversion is a metabolic phenomenon, competing with other pathways of inversion. This is demonstrated by the non-steroselective metabolism of ibuprofen to 2-[4-(2-carboxypropyl)phenyl]propionic acid (Wechter et al., 1974; Kaiser et al., 1976). Studies involving ibuprofen metabolites have been published by Mills et al. (1973), Brooks and Gilbert 1976), and Giachetti (1985).

An additional factor responsible for the predominance of S-ibuprofen in urine may be the preferential formation of the S-ibuprofen glucuronide. It has been suggested (Lee et al., 1985) that stereoselective glucuronidation may be the result of the preferential enzymatic hydrolysis of one of the ester conjugates. This proposition is an extension of the futile cycle hypothesis involving clofibric acid in which the net plasma clearance is determined by conjugation and competition between renal clearance and hydrolytic clearance of the glucuronide (Meffin et al., 1983a, 1983b). However, it may be suggested that such a rationale for the stereoselective glucuronidation of ibuprofen is probably applicable only in the presence of renal dysfunction. It is more likely that stereoselectivity in the urinary excretion of ibuprofen is due to the greater susceptibility of the S-(+)-isomer to glucuronidation.

Enantioselective non-linear protein binding of racemic ibuprofen may contribute to the stereoselective excretion of ibuprofen isomers as well as inadvertently influence the overall stereoselectivity (including chiral inversion). This observation may be explained by the following factors: (1) it has been conclusively demonstrated that stereoselective protein binding is responsible for overall stereoselectivity in studies with hydratropic acid (the fundamental structural unit of 2-APAs) (Yamaguchi and Nakamura, 1985) and indoprofen (Tossolini et al., 1974; Tamassia et al., 1984; Bjorkman, 1985); (2) for basic drugs, such as propranolol, stereoselective binding to human plasma α_1-acidglycoprotein and albumin has been demonstrated (Walle et al., 1983). This may be of some significance as only a small proportion of basic drugs are protein bound (Reidenberg and Drayer, 1984); (3) since albumin is inherently chiral, it has been utilized as a chiral stationary phase in HPLC (Allenmark et al., 1982, 1983, 1984a, 1984b; Allenmark and Bomgen, 1982; Fitos et al., 1983; Miklos and Fitos, 1983). The protein to which acids are extensively bound is, in fact, albumin (Wanwinolruk et al., 1982); (4) there may be a relationship between non-linear protein binding and enantiomer-enantiomer interactions. Williams and Lee (1985) have described a similar concept in terms of a drug-drug interaction in which the drugs were competing for the same protein binding site.

The implications of such a relationship may be of importance; this is particularly true when considering the observed non-linearity in the non-stereoselective pharmacokinetics of ibuprofen as a consequence of non-linear protein binding (Lockwood et al., 1983; Grennan et al., 1983). Enrichment of S-(+)-ibuprofen as a result of inversion as well as the presence of uninverted R-(-)-ibuprofen (which competes for protein binding sites) may magnify the non-linearity of protein binding for the S-(+)-isomer. As a result, a greater proportion of unbound S-ibuprofen would be available for glucuronidation and consequently account for the stereoselective excretion of the enantiomers.

The role of protein binding may be evaluated with some degree of certainty by relevant in vitro and liver perfusion investigations along with simultaneous in vivo studies.

Ibuprofen was not investigated further since its stereoselective distribution in humans (Vangiessen and Kaiser, 1975; Kaiser et al., 1976; Lee et al., 1985), the mechanisms of inversion (Wechter et al., 1974; Lee et al., 1985), and the species variation associated with disposition of the enantiomers (Crowther et al., 1984; Cox et al., 1985) had been studied extensively.

3.5.2.0.0.0 PHARMACOKINETICS OF TIAPROFENIC ACID ENANTIOMERS IN HUMANS

The levels of steady state tiaprofenic acid enantiomers were measured from the plasma and urine specimens obtained from four arthritic subjects and synovial concentrations following a single 200 mg dose to six other subjects (see Section 2.9.2.3.1.0, Materials and Methods).

Tiaprofenic acid concentrations in the biological samples were determined from standard curves obtained by spiking blank samples of plasma, urine and synovial fluid with known amounts of racemic tiaprofenic acid. The concentrations thus obtained were 0.25, 0.5, 1, 2, 5, 10 and 20 ug/mL of racemate. In all specimens excellent linearity was observed ($r^2 = 0.994 - 0.997$) for three sets of standard solutions. A typical calibration curve could be described by $y = 0.106x + 0.0001$ and the concentrations of the enantiomers in the specimens were calculated using this equation. The CV for corresponding enantiomer concentrations (one-half of racemate) in all three specimens was less than 10%.

As determined from the GC peak area responses of the diastereoisomers, 1-hydroxybenzotriazole had no apparent effect on the reaction between tiaprofenic acid and amphetamine.

The efficiency of the toluene extraction procedure, as determined for 5 ug of racemate, was 68.3 +/- 1.43% (n=5). Attempts to improve the extraction efficiency by the addition of isopropanol as a co-solvent (Litowitz et al., 1984) resulted in the concomitant extraction of trace amounts of water which had a detrimental effect on the CDI-mediated coupling reaction.

The identities of the tiaprofenic acid enantiomers, extracted from either spiked or subject samples, were confirmed as the respective S-(+)-AM diastereoisomers by electron-impact and chemical ionization GC/MS. The quasi-molecular ion was evident at m/z 378. In the electron-impact mass spectrum (Table 15) the molecular ion (m/z 377) was not observed. However, the characteristic [M-117]+ fragment, arising from the expulsion of a C_9H_9 radical from the amphetamine portion of the molecular ion, was present in low abundance. Other fragments included m/z 216 as the result of a McLafferty rearrangement and expulsion of $C_6H_5CH_2CH(CH_3)N=C=O$ (161 amu) from the molecular ion, and m/z 284 (M-91) resulting from the loss of a benzyl radical. Loss of $C_6H_5CH_2CH(CH_3)NH$ (134 amu) and $C_6H_5CH_2CH(CH_3)NHCO$ (162 amu) radicals gave fragments m/z 243 and m/z 215 respectively. Additional fragments from the NSAID portion of the molecular ion included m/z 187

$[C_{11}H_7SO]^+$ and m/z 105 $[C_6H_5CO]^+$. Fragments m/z 91 $(C_6H_5CH_2^+)$, 117 $[C_6H_5C{=}CHCH_3]^+$ and 118 $[C_6H_5CH{=}CHCH_3]^+$ arise from the amphetamine portion of the molecular ion.

Chromatograms of plasma and urine samples from subjects following ingestion of 200 mg tiaprofenic acid, three times daily, as well as synovial fluid samples following a single 200 mg dose are depicted in Figures 76-78. No significant differences were observed in the concentrations of the enantiomers. Indeed, the time course of the enantiomers were superimposable (Figure 79, Table 18). The isomers undergo rapid GI absorption with peak plasma concentrations achieved between 0.5 - 2 hours. The terminal half lives of approximately 2 hours indicate rapid elimination. The observed small Vd/F values indicate extensive plasma protein binding for the enantiomers. These observations are in agreement with studies utilizing non-stereospecific assays (Pottier et al., 1977; Jamali et al., 1985).

The similar disposition kinetics observed for tiaprofenic acid enantiomers suggests that these isomers not only have identical elimination kinetics, but that like indoprofen (Tamassia et al., 1984) they may present an exception to the observation that most 2-arylpropionic acids undergo inversion. As a consequence, there would be no ambiguity in generating pharmacokinetic data based on measurements of total drug concentration (R- plus S-) by non-stereospecific methods (Jamali et al., 1984). Although chiral inversion was not observed when racemic tiaprofenic acid was administered, definitive confirmation requires the administration of individual isomers. However, it is reasonable to suggest that in humans, metabolic chiral inversion does not occur, or the extent of inversion is insignificant. A drug with slow metabolic inversion and rapid elimination may also display time courses similar to those observed for TA. For example, it has been reported (Bopp et al., 1979) that the inversion half-life of R-benoxaprofen was approximately 108 hours whereas its elimination half-life was about 30 hours. As a result, substantial quantities of the R-isomer may be eliminated before significant inversion can occur.

A possible relationship between biliary excretion and chiral inversion should also be considered. Animals such as rats and dogs which are good biliary excretion models (Klaasen and Watkins, 1984) are also excellent inverters of APAs (Lan et al., 1976; Kripalani et al., 1976; Tanaka and Hayashi, 1980). As a consequence the rapid urinary excretion of TA might account for the lack of inversion in humans.

It should be noted that with benoxaprofen the overall stereoselectivity is due only to chiral inversion since the elimination rates of the enantiomers are the same (Simmonds et al., 1980). It should also be noted that for indoprofen and carprofen, which do not undergo significant chiral inversion, the overall pharmacokinetic stereoselectivity is due to differential protein binding and excretion rates of the isomers in the case of indoprofen (Tosolini et al., 1974; Tamassia et al., 1984; Bjorkman, 1985) and preferential excretion of the R-isomer in the case of carprofen (Stoltenborg et al., 1981).

The urinary excretion data suggests that not only are the excretion rates of conjugated tiaprofenic acid enantiomers similar (Fig. 56) but the cumulative amounts excreted in the urine are also virtually identical (Table 18). These observations support the conclusion that the excretion of tiaprofenic acid enantiomers is not stereoselective.

Tiaprofenic acid is present in significant amounts in the synovium (synovial fluid to plasma concentration ratios of approximately 0.2 - 2.0) (Daymont and Herbert, 1982). Synovial fluid is not only a possible site of action for NSAIDs but it also provides an insight into the extravascular pharmacokinetics of a drug (Wallis and Simkin, 1983). Since equivalent concentrations of the optical isomers were observed in synovial fluid (Table 19), the extravascular distribution of the enantiomers is apparently also non-stereoselective.

In addition to metabolic chiral inversion, stereoselective metabolism may influence the kinetic disposition of enantiomers. Metabolism does not play an apparent role in the stereoselective disposition of indoprofen and benoxaprofen; negligible quantities of metabolites (indole ring-hydroxylated derivatives) are formed from indoprofen (Fucella et al., 1973) while with benoxaprofen, metabolites other than conjugates have not been observed in humans (Bopp et al., 1979).

No effort was made to quantitate or determine the stereochemistry of the known metabolites of tiaprofenic acid (hydroxylation of the phenyl ring and reduction of the carbonyl group) (Pottier et al., 1977; Jamali et al., 1985). However, since less than 5% of tiaprofenic acid is metabolized through these pathways (Pottier et al., 1977; Jamali et al., 1985), it would be anticipated, as with indoprofen (Tossolini et al., 1974; Tamassi et al., 1984; Bjorkman, 1985) and benoxaprofen (Bopp et al., 1979), that these metabolites would not significantly affect the overall pharmacokinetics of the drug.

Metabolic chiral inversion is expected to be competitive with other routes of metabolism. If there is no inversion, or if the inversion rate is slow, either isomer should be equally available for other metabolic processes. This concept is illustrated by the non-stereoselective metabolic formation of 2-[4-(2-carboxypropyl)phenyl]-propionic acid from ibuprofen (Kaiser et al., 1976). In addition, it is expected that the stereoselectivity of metabolic enzymes will be dependent, in part, on the proximity of a drug's chiral center to the site of metabolism (Williams and Lee, 1985). In the case of tiaprofenic acid, since the sites (para-position of the phenyl ring and the ketone carbonyl) are relatively far-removed from the asymmetric carbon, metabolism is probably non-stereoselective.

The issue of pharmacological stereoselectivity of tiaprofenic acid enantiomers has not been addressed in the literature. It is feasible, that like other 2-arylpropionic acids, only one of the isomers is active while the other is simply isomeric ballast. If anti-inflammatory activity is due to one enantiomer, and as chiral inversion was not observed, the availability of tiaprofenic acid as the pharmacologically active isomer, rather than the racemate, may represent a therapeutic advantage. By comparison it has been suggested that naproxen is marketed as the S-enantiomer to avoid "the possibility of undesirable side effects and the renal burden of clearing 'inactive' R-naproxen" (Williams and Lee, 1985). Furthermore, pharmacokinetic non-stereoselectivity concurrent with pharmacological stereoselectivity is a known phenomenon (e.g. fenfluramine in man; Caccia et al., 1982). The observations with tiaprofenic acid with respect to the pharmacokinetic non-stereoselectivity of the enantiomers may be a further example.

3.5.3.0.0.0 STEREOSELECTIVITY IN THE DISPOSITION AND METABOLISM OF ETODOLIC ACID

For pharmacokinetic studies with etodolic acid, blood and urine samples were obtained from two subjects (see Section 2.9.2.4.1.0, Materials and Methods).

Gas chromatographic peaks representing diastereoisomers of (+)- and (-)-etodolac eluted at 17.5 and 19.8 min respectively (Fig. 80). The derivatized internal standard (S-naproxen) had a retention time of 12.5 min. The MS fragmentation patterns for each diastereoisomer prepared from authentic standards were identical to the respective isomers observed in the samples. Characteristic fragment ions of significant abundance (Rel. Abund. >35%) were present in both spectra and were readily identified. Fragmentations were predictable by locating the initial charge on the ring oxygen atom of etodolac; elemental compositions of ions were confirmed by high resolution mass spectrometry (Fig. 81).

The calibration curves for each derivatized enantiomer were linear within the examined concentration range. Typical curves for the (+)- and (-)-isomers could be described by $y=-0.0809x + 0.2789$ and $y=-0.0901x + 0.2752$ respectively. The coefficient of correlation for either enantiomer extracted from plasma was 0.997 and from urine, 0.992. The minimum quantifiable concentration of each derivatized enantiomer was 50 ng/ml of plasma or urine with an on-column detection limit of 0.5 ng. The observed coefficients of variation were always less than 8% (n=3). Sensitivity, and hence the slope of the calibration curve, was found to be very dependent upon the age and condition of the rubidium sulfate bead in the nitrogen-phosphorus detector.

Prior to extraction, the etodolac-containing sample of urine or plasma was basified (NaOH) and washed with diethyl ether. This procedure removes endogenous amines which might otherwise compete with the derivatizing agent in the coupling reaction. In urine samples, the NaOH also hydrolyzes ester conjugates. It is apparent from Figure 82 that no significant amount of unchanged etodolac was present in urine. As with tiaprofenic acid (Jamali et al., 1984, 1985), the addition of 50 ul of 1 M NaOH followed by vortexing for 0.5 min effectively hydrolyzed the conjugates.

Following acidification of the samples, the best recovery of etodolac enantiomers was obtained when toluene was used for extractions. This solvent minimized the carry-over of water which has a deleterious effect on the coupling reaction. The percentage recovery from plasma of (+)- and (-)-enantiomers (2.5 ug of each) was 66.50 +/- 0.04 and 65.83 +/- 0.03 (n=5) respectively. Attempts to improve recovery by adding isopropanol as a co-solvent (Litowitz et al., 1984) resulted in the concomitant extraction of trace amounts of water as well as unidentified endogenous components.

The plasma concentrations of the enantiomers seem to rise and fall in a parallel fashion (Fig. 83). Both enantiomers reach their maximum concentrations within 1 - 1.5 hours after administration. The appearance of a second peak in the plasma concentration-time curves of both subjects may indicate enterohepatic recirculation of the drug (Cayen et al., 1981). Interestingly, the plasma concentrations of the more active (+)-isomer were consistently lower than those of the less active (-)-isomer. This observation with the arylacetic acid derivative, etodolac, is contrary to what is commonly reported for the 2-arylpropionic acid class of NSAIDs; the more active isomers are usually predominant in plasma (Hutt and Caldwell, 1983; Williams and Lee, 1985).

During the 48 hour collection period, subject M excreted 9.05% and 5.65% of the dose as readily hydrolyzable conjugates of the (+)- and (-)-isomers, respectively. Subject F excreted 9.96% and 6.52% of the dose as conjugated (+)- and (-)-isomers, respectively, during one dosing interval. The more intensive formation, and consequently urinary excretion, of the conjugated (+)-isomer as compared to the (-)-isomer, may partly explain the observed lower plasma concentration of the (+)-isomer conjugate. The estimated pharmacokinetic parameters calculated from the above analyses are shown in Table 20.

During the course of this study three urinary metabolites of etodolac were observed. The GC/MS properties of the metabolites were consistent with (1) a dehydrogenated product, (2) N-methyletodolac, and (3) a phenolic metabolite (Fig. 84).

Both enantiomers (as derivatives) of the dehydrogenated metabolite are evident in Figures 85 and 86 (the etodolac diastereoisomers as well as one of the N-methyletodolac diastereoisomers are also evident in Figure 86). If the relationship between the order of elution and optical activity of etodolac is extended to the dehydrogenated metabolite, the first eluting isomer would be dextrorotatory. From Figures 85 and 86, it is apparent that formation of the (+)-dehydrogenated metabolite is favored over that of the (-)-isomer. Based upon a comparision of

(-)-isomer. Based upon a comparison of the fragmentation patterns of etodolac and this metabolite (Fig. 81), the product was tentatively identified as 8,9-dehydroetodolac. The metabolic origin of this compound was determined by control studies (i.e. this product was not observed in standard etodolac samples) and confirmed by selected ion monitoring (molecular ions of 404 and 402 for etodolac and the metabolite respectively).

However, with the isolation of 8-hydroxyetodolac as a metabolite (Ferdinandi et al., 1985) the metabolic origin of 8,9-dehydroetodolac is open to question. It is plausible that 8,9-dehydroetodolac is a 'work-up'-induced dehydrogenated product arising from the dehydration of the 8-hydroxy metabolite. Due to the unavailability of authentic standards it was not possible to conclusively determine whether the dehyrogenated compound was, in fact, a true metabolite.

The structure of the N-methyl metabolite was confirmed (Figs. 81, 86, 87) by comparison with an authentic synthetic product. As only one of the enantiomers was observed, the optical rotation could not be assigned.

A phenolic derivative of etodolac was also isolated as a major metabolite. Based upon proton NMR (Fig. 89) and MS (Figs. 90-94) data, the product was tentatively identified as the 7-hydroxy metabolite. The formation and structure of this metabolite has been recently reported (Ferdinandi et al., 1986) and is consistent with the data from these laboratories. Interestingly, this metabolite is observed (chromatographic analysis) only after trifluoroacetylation of the diastereoisomeric derivatives (Fig. 84, 95). Metabolism appears to favor formation of the (+)-isomer (again the sign of rotation is assigned on the basis of comparison with the elution order of etodolac diastereoisomers).

These metabolic studies also offer a partial explanation for the observed stereoselectivity in the disposition of etodolac isomers. Preferential elimination (through biotransformation and excretion) may be the principal reason for the observed relatively lower plasma levels of (+)-etodolac in humans.

3.6.0.0.0.0 CONCLUSION

During the course of these studies the importance of stereochemistry on drug disposition was demonstrated through the use of various models.

The versatility of C. echinulata was successfully exploited for oxidative metabolism of two chemically different substrates. The stereospecific sulfoxidation of CS and regioselective p-hydroxylation of pre-prenalterol demonstrated the usefulness of a microbial model of mammalian metabolism. This model was further utilized for the preparative biosynthesis of racemic prenalterol, thereby providing a potentially attractive alternative to exclusive chemical synthesis.

In a study involving the GC resolution of amphetamine-N-TPC diastereoisomers, the contributions of the amide proton and non-bonding interactions in chiral discrimination were demonstrated. The GC method was used successfully to investigate the stereoselective N-deethylation of R-(-)-fenfluramine in the rat.

The development of a sensitive GC method for the stereospecific analysis of arylalkanoic enantiomers resulted in its application to investigations on the disposition of ibuprofen, tiaprofenic acid and etodolic acid in humans. These NSAIDs may be closely related in terms of pharmacological activity but they differ dramatically with respect to their stereoselective dispositions.

The stereoselectivity observed with ibuprofen may be attributed, in part, to the unidirectional inversion of the distomer to eutomer. With tiaprofenic acid, stereoselectivity was not apparent. Chiral inversion was not observed and therefore this NSAID is an exception to the general notion that "inversion must be assumed to occur in the absence of proper contradictory evidence" (Hutt and Caldwell, 1983). The stereoselective disposition of etodolac is novel in the arylalkanoic group of NSAIDs in that plasma levels of distomer were consistently higher than eutomer. Rapid clearance of the eutomer may account for this observation.

The results from the NSAID studies highlight the importance of developing sensitive and enantiospecific analytical methods for clinical investigations. In rheumatology, the issue of bio- and therapeutic equivalence and the absence of a clear relationship between blood levels and therapeutic efficacy are topics of current research interest. Significant efforts have been made to address these issues, but only recently has the importance of stereoselective disposition (including chiral inversion) been recognized. In this regard, volumes of pharmacokinetic data have been generated without due consideration of the importance of stereochemistry in drug action. It is apparent from the studies reported in this dissertation that an analytical procedure for the resolution and measurement of the individual enantiomers, present in biological fluids, is essential for the proper interpretation of pharmacokinetic data obtained following the administration of drugs as racemates.

TABLES

TABLE 1 DIRECT GC RESOLUTION

(+/-)-SELECTAND	SELECTOR(CSP)	REFERENCES
α-hydroxy acids	Chirasil-Val	Koenig & Sievers 1980, Koenig et al 1980, Wang et al 1984, Koppenhoefer 1985.
α-halocarboxylic acids	various amino acid derived diamide phases	Chang et al 1984, Watabe 1985.
amino acids	Chirasil-Val	Frank et al 1978c, Gerhardt et al 1984.
	XE-60-L-valine *t*-butylamide	Abe et al 1983, Konig et al 1983.
	N-lauroyl-L-valine *t*-octylamide, N-docosanoyl L-leucine *t*-butylamide	Charles & Watabe 1984.
	Chirasil-Val	Haegle et al 1983.
sulfhydryl amino acids	Chirasil-Val	Bayer et al 1985.
β-adrenoceptor blocking agents	XE-60-L-valine R-α-phenyl ethylamine	Konig & Ernst 1983, Konig et al 1984a, Konig et al 1986.
antiarrhythmic agents	Chirasil-Val	McErlane & Pillai 1983; Antonsson et al 1984.
anticonvulsants	Chirasil-Val	Wedlund et al 1984.

TABLE 2 INDIRECT GC RESOLUTION

(+/-)-SELECTAND	SELECTOR(CSP)	REFERENCES
α-hydroxy acids	MPTA	Beneytout et al 1986
amino acids	various perfluoroacylated analogues of L-prolyl chloride	Iwase 1974a, 1974b; Iwase & Murai 1974a, 1974b.
	(+)-3-methyl -2-butyl alcohol	Konig et al 1977b.
	2-butanol	Anders & Goeran 1984.
antiarrhythmic agents	MPTA-Cl	Sedman & Gal 1984.

TABLE 3 DIRECT HPLC RESOLUTION

(+/-)-SELECTAND	SELECTOR(CSP/CMP)	REFERENCES
mandelic acids	cyclodextrin (CMP)	Debowski et al 1983
	cyclodextrin (CSP)	Gubitz et al 1984b
carboxylic acids	quinine (ion-pair)	Petterson 1984
amino acids	2,2,2, -trifluoro 1-(9-anthryl) -ethanol	Pirkle & Tsiporous 1984, Pirkle & Hyun 1984
	S-1-α-naphthyl ethylamine	Oi et al 1983a
β-adrenoceptor blocking agents	R-N-3,5 dinitrobenzoyl phenylglycine (covalently bonded Pirkle column)	Wainer & Doyle 1984c
	α_1-acid glycoprotein	Hermansson 1985
	(+)-camphorsulphonic acid (ion-pair)	Petterson & No 1983
sympathomimetic agents	R-N-3,5-dinitro benzoyl phenylglycine (covalently bonded Pirkle column)	Wainer et al 1983
	(+)-di-nbutyl tartarate	Petterman and Stuurman 1984
	(+)-tartaric acid	Kicinsky and Kettrup 1985
antiarrhythmic agents	α_1-acid glycoprotein	Hermansson 1984b
anxiolytics	3,5-dinitro benzamides of R-phenylglycine and R-leucine	Pirkle & Tsipouras 1984
	human serum albumin	Fitos et al 1983, Fitos & Mikos 1983
	α_1-acid glycoprotein (CSP/CMP)	Hermansson 1983, 1984a, 1984b

sedatives	cellulose triacetate	Blaschke 1984, Blaschke & Hildegunde 1984
local anaesthetics and antihistaminics	α_1-acid glycoprotein	Hermansson 1983, 1984a
antineoplastics and antimetabolites	Cu(II)-L-phenyl alanine (CMP)	Cramer et al 1984
antiherpes agents	Cu(II)-L-phenyl alanine (CMP)	Forsman 1984.

TABLE 4 INDIRECT HPLC RESOLUTION

(+/-)-SELECTAND	SELECTOR (CSP/CMP)	REFERENCES
amino acids	GITC	Nimura et al 1984
	t-butyloxycarbonyl L-cysteine	Buck & Krummen 1984
	N-acetyl-L-cysteine	Aswad 1984
	N-t-butyl-oxycarbonyl L-leucine-O-succinimide	Chen et al 1984, Allison et al 1984
oxfenicine	R(+)MPTA anhydride	Coleman 1983
β-adrenoceptor blocking agents	(R)-(-)-phenyl-ethyl isocyanate	Gulaid et al 1985
	(R)-α-methylbenzyl isocyanate	Gal 1984
	(R)-1-(1-naphthyl)-ethyl isocyanate	Gubitz et al 1984a
	R,R or S,S tartaric acids	Linder et al 1984
	as diastereomeric glucuronide cojugates	Wilson & Thompson 1984
sympathomimetic agents	GITC	Gal 1984, Allgire et al 1985
	(R)-α-methylbenzyl isocyanate	Gal & Sedman 1984
	(-)-camphonyl chloride	Selvig et al 1983 Ruud-Christensen & Salvesen 1984
antiarrhythmic agents	(R)-(-)-O-methyl-mandelic acid	Hoffman et al 1984
	GITC	Grech-Belanger et al 1985
anticoagulants	(-)menthyl chloroformate	Jeyaraj & Porter 1984
	carbobenzyloxy L-proline	Banfield & Rowland 1983, 1984
anxiolytics	as diastereomeric glucuronide conjugates	Masher et al 1984, Hale & Poklis 1984

TABLE 5 CHROMATOGRAPHIC RESOLUTION OF NATURAL AGENTS

AGENTS INVESTIGATED	COMMENTS
Menthol isomers (Haut & Core 1981)	A native diastereomer therefore separable on normal phase HPLC.
Carbohydrates (Schweer 1982)	Enantiomers separated as (-)-menthyloxime perfluoroacylates on OV-225 phase.
Some insecticides containing sulfur or phosphorus asymmetric centres (Okamoto et al 1984)	Resolved on (+)-poly(triphenylmethyl)-methacrylate as a CSP by liquid chromatography.
R,S-abscisic acids (Vaughan et al 1984)	Reduction of acid functionality yields a diastereoisomer separable by reverse phase HPLC.
Chiral insect pheromones (Weber & Schurig 1984, Schurig & Weber 1984)	An overview on applications of complexation gas chromatography with enantioselective transition metal β-ketoenolates.
Fungicides (Clark & Dean 1985)	Separation on Chirasil-Val GC phase
Carotenoids (Maoka et al 1985, Ikuno et al 1985)	Direct separation on a Sumipac OA-2000 phase by liquid chromatography.
Terpen-4-ol (Schurig 1985)	Direct separation by complexation GC.
Citronellal (Taylor & Schreck 1985)	A comparative study of GC-CSPs.

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TABLE 6 SELECTED LITERATURE REFERENCES DEALING WITH THE RESOLUTION OF ISOMERS

CHROMATOGRAPHIC METHOD	COMMENTS (NUMBER OF REFERENCES)
GC (Gil-Av & Nurok 1974)	A review on resolution of diastereoisomers. Contents comprise classes of compounds resolved, procedures used, mechanisms of resolution and applications.
GC (Beitler & Feibush 1976)	Discussion of mechanistic aspects (including thermodynamics) of chiral resolution on L-valine-derived CSPs.
GC (Konig 1983)	Review of various thermostable CSPs, which includes derivatization procedures for the purposes of direct resolution.(70)
GC (Schurig 1984)	A review of resolution of enantiomeric mixtures by GC on peptide-derived and metal-containing CSPs.(98)
GC (Liu & Ku 1983)	A concise overview of GC-CSPs, that includes the mechanistic aspects of separation.(53)
GC (Schurig 1984)	An extensive review of resolution of enantiomers on optically active amino acid and peptide selectors.(188)
GC (Gil-Av 1985)	Primarily a review of development of direct chromatographic resolution methods in the author's laboratory. Various selector types and mechanisms involved in chiral recognition are described.(67)
GC (Koppenhoefer & Bayer 1985b)	Critical review of chiral recognition on Chiral-Val phase.(75)
HPLC (Krull 1979)	Describes the various HPLC approaches that have resulted in either partial or complete resolution of enantiomers.(138)

HPLC (Blaschke 1980)	Discussion of various optically active adsorbents available for resolving racemates. Mechanisms are explained with the help of model compounds.(68)
HPLC (Davankov et al 1983)	Primarily a review on direct and indirect resolution by HPLC. Also included is a brief reference to other types of chromatographic methods.(157)
HPLC (Pirkle & Finn 1983)	Comprehensive review of direct and indirect methods of resolution by HPLC. Also included are various types of diastereoisomers, natural and synthetic CSPs.(86)
HPLC (Armstrong 1984)	The HPLC-CSPs have been conveniently classified into six groups to discuss mechanisms, strengths and limitations.(63)
HPLC (Cacchi et al 1984)	Reviews the nature and separation mechanisms of the CSPs, including ligand exchange chromatography.(37)
HPLC Pirkle et al 1984, Pirkle & Tsiporous 1984, Pirkle & Hyun, 1984)	Series of papers illustrating the role of numerous chiral mechanisms on commercially available CSPs developed by W. H. Pirkle. Relates an understanding of the mechanistic aspects to the effective design and use of CSPs.
HPLC (Wainer & Doyle 1984a)	An review of Pirkle type HPLC-CSPs for resolution of racemic pharmaceuticals. A concise but clear explanation of the fundamentals of chiral recognition.(33)
HPLC(LEC) (Wernicke 1985)	An excellent reference for understanding mechanistic aspects, and particularly the role of mass-transfer in HPLC.
GC/HPLC (Lochmuller & Souter 1975)	Covers the various mechanisms of direct and indirect resolution by chromatography.

GC/HPLC (Oi 1984)	A review of the various methods developed in the author's laboratory.(43)
GC/HPLC (Mason 1986)	Basic overview emphasizing the importance of recognizing the racemic nature of drugs and the chromatographic methods available for chiral separation.(14)
General (Wilson et al 1971)	A general review of important and practical methods of resolution including chromatography.(384)
General (Yoneda 1985)	Elucidates the mechanisms of separation of complexes with emphasis on chiral discrimination.(73)
General (Testa 1986)	A review of various stereospecific chromatographic methods employed for metabolism studies.(57)
Preparative (Wilen 1977)	Review on resolutions involving crystallization.(43)

TABLE 7 BOOKS AND SPECIAL ISSUES

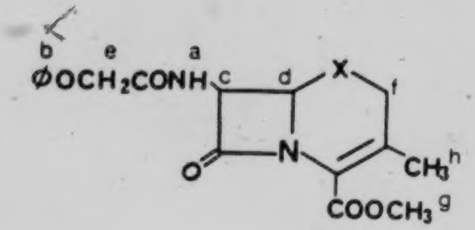
REFERENCE	COMMENTS
Souter, R. W., (1985)	Deals with currently available techniques for resolving stereoisomers by chromatography. Includes 800 references covering all the disciplines of optical resolution by chromatographic means. Applications have been tabulated which facilitates the use of this excellent reference book.
J. Liquid Chromatogr. 2(8):1063-1254 (1979)	Special issue on liquid chromatographic separation of enantiomers (pp:1063-1095), diastereoisomers (pp:1229-1250) and configurational isomers.
J. Liquid Chromatogr. 9(2+3):241-700 (1986).	Special issue on "Optical Resolution by Chromatography". Covers all aspects of chiral resolution by liquid chromatography.

TABLE 8 KINETIC PARAMETERS FOR MICROBIAL METABOLISM OF 7-((PHENOXY)ACETAMIDO)CEPHALOSPORANIC ACID, METHYL ESTER (CS) TO ITS SULFOXIDE METABOLITE (CSO)[1].

Concn. CS ug/mL	T1/2 CS	T1/2 CSO	KE CS	KE CSO	KF CSO	Vmax CS	Kmax CS
25	1.08	1.20	0.64	0.58	0.9	88.50	60.00
100	0.96	1.18	0.72	0.59	1.3	99.00	52.30
200	1.11	3.06	0.63	0.24	-	114.6	61.40
400	1.35	2.83	0.51	0.25	-	127.4	58.50

[1]based on mean of 3 values.

TABLE 9 PROTON NMR CHEMICAL SHIFTS (PPM) OF 7-[(PHENOXY)ACETAMIDO]CEPHALOSPORANIC ACID, METHYL ESTER (CS)& ITS R-SULPHOXIDE (CSO)

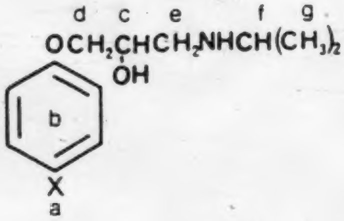

b
e
a
ΦOCH₂CONH
c
d
X
f
N
O
CH₃ h
COOCH₃ g

X	a	b	c	d	e	f	g	h
S (CS)	9.00	7.05	5.62	5.10	4.70	4.30	3.84	2.07
SO (CSO)	8.58	7.00	6.08	5.38	4.70	4.30	3.84	2.06

TABLE 10 SOME BASIC KINETIC PARAMETERS OF 1-PHENOXY-3-(ISOPROPYLAMINO)-2-PROPANOL (PP) METABOLISM BY *C. echinulata*

CONCN. PP ug/mL	β PP ug/mL.day	T1/2 PP days	% yeild Prenalterol
100	0.509	1.36	90.61
200	0.388	1.78	96.32
400	0.543	1.27	86.42

TABLE 11 PROTON NMR CHEMICAL SHIFTS (PPM) OF PRE-PRENALTEROL (PP) & PRENALTEROL (P)


d c e f g
OCH2CHCH2NHCH(CH3)2
OH
b
X
a

X	a	b	c	d	e	f	g
H PP	-	6.95- 7.30	3.20	3.00	1.88t	1.75	1.30
OH P	7.90	6.30	2.95	2.85	1.80	1.65	1.12

TABLE 12 RETENTION TIMES (RT) AND SEPARATION FACTORS (α) OF SEVERAL AMPHETAMINE-N-TPC DIASTEREOISOMERS

AMINE	RT min (-)- *isomer*	RT min (+)- *isomer*	α
(+/-)-Amphetamine	11.82	12.45	1.053
(+/-)-Methylamphetamine	16.15	16.81	1.041
(+/-)-*n*-Butylamphetamine	24.03	24.99	1.035
(+/-)-Fenfluramine	17.29	18.11	1.047
(+/-)-Norfenfluramine	12.71	13.13	1.033
(+/-)-Fluoroamphetamine	13.18	13.75	1.044
(+/-)-*p*-Chlorofluoroamphetamine	20.96	22.96	1.093
(+/-)-Tranylcypromine	18.62	20.24	1.087
(+/-)-Fluorotranylcypromine	17.69	18.98	1.073

TABLE 13 COMPARATIVE PERFORMANCES OF CAPILLARY AND PACKED COLUMNS[1]

AMINE	RT[2] min (-)- *isomer*	RT[2] min (+)- *isomer*	RT[3] min (-)- *isomer*	RT[3] min (+)- *isomer*	α^2	α^3
(+/-)- Amphetamine	11.82	12.45	4.95	5.45	1.053	1.096
(+/-)- Methyl amphetamine	16.15	16.81	13.50	14.50	1.041	1.074
(+/-)- Fluoro amphetamine	13.81	13.76	8.81	9.49	1.044	1.077

[1] Capillary column 0.323mm, Packed column HETP = 4.048mm.

[2] RT = Retention time on capillary column.

[3] RT = Retention time on packed column.

α^2 = Separation factor on capillary column.

α^3 = Separation factor on packed column.

TABLE 14 GC RETENTION TIMES (MIN) OF NSAID DIASTEREOISOMERS USING S-(+)-AMPHETAMINE (S*) OR R-(-)-AMPHETAMINE (R*) AS RESOLVING AGENTS

NSAID	S;S*[1]	R;S*[1]	R;R*[1]	S;R*[1]
Ibuprofen	17.11	17.46	17.10	17.48
Ketoprofen	12.80	13.40	12.84	13.47
Naproxen	12.57	12.89	12.62	12.80
Fenoprofen	19.18	19.31	19.13	19.46
Flurbiprofen	13.14	13.57	13.15	13.51
Pirprofen	9.73	10.01	9.74	10.01
Tiaprofenic acid[2]				
Etodolac[2]				

[1]Configuration of the NSAID;diastereoisomer, [2]see text.

TABLE 15 PROPOSED MASS-SPECTRAL FRAGMENTS (% RELATIVE ABUNDANCE) OF (R,S)-2-ARYLPROPIONIC ACID-α-(R or S)-METHYLBENZENEETHANAMINE DIASTEREOISOMERS

APAs	I	II	III	IV	V	OTHERS
Ibuprofen	323 (1)	232 (74)	161 (100)	162 (16)	206 (8)	91 (50)
Ketoprofen	371 (0.5)	280 (100)	209 (82)	210 (28)	254 (8)	105(93) 77(12) 237[1](10)
Naproxen	347 (21)	256 (14)	185 (100)	186 (30)	230 (00)	171(14) 141(22) 128(60) 115(14)
Fenoprofen	359 (1.5)	268 (84)	197 (100)	198 (42)	242 (6)	241 (9)
Flurbiprofen	361 (14)	270 (77)	199 (100)	200 (36)	244 (6)	243(12) 207(27)
Pirprofen	368/370 (45,17)	277/279 (6,1.5)	206/208 (100,26)	207/209 (17,4)	251/253 (00)	234/236[1] (5,1.2) 170[2](26) 332[3](11)
Cicloprofen	355 (15)	264 (31))	193 (100)	194 (33)	238 (00)	237[4](11) 178(38) 165(33)
Tiaprofenic acid	377 (1)	286 (32)	215 (44)	216 (100)	260 (7)	105(73) 243[1](34)
IS[5]	283 (13)	192 (47)	121 (100)	122 (17)	166 (4)	165(8) 148(3)

[1] =M - $NHCH(CH_3)CH_2Ph$

[2] =206/208 - HCl

[3] =M - HCl

[4] =M - $CH_2CH=CHPh$

[5] =p-Methoxyphenylacetic acid.

I=M

II=M - 91

III=M - $CONHCH(CH_3)CH_2Ph$

IV= McL

V=M - PhC_3H_4

TABLE 16 BIOAVAILABILITY OF IBUPROFEN (MOTRIN) 600mg TABLETS

SUB No.	Tmax S h	Tmax R h	Cmax S mg/L	Cmax R mg/L	Cmax S/R	AUC^{12} S mg.h/L	AUC^{12} R mg.h/L	AUC^{12} S/R
1	1.00	1.00	16.37	16.65	0.98	63.76	44.66	1.43
2	1.00	1.00	31.13	28.43	1.09	94.96	67.23	1.41
3	1.00	1.00	27.24	28.62	0.95	188.19	101.59	1.85
4	2.00	1.00	22.97	19.85	1.16	85.48	52.91	1.62
5	3.00	3.00	26.39	16.52	1.60	128.21	65.25	1.96
6	2.00	3.00	63.60	56.74	1.12	162.43	118.33	1.37
7	1.00	1.00	22.00	18.58	1.18	67.42	39.41	1.71
8	1.00	1.00	28.56	26.96	1.06	143.11	98.32	1.46
9	.50	.50	25.00	27.00	0.93	96.75	88.55	1.09
MEAN	1.39	1.39	29.25	26.59	1.10	114.48	75.14	1.52
S.D.	00.78	00.93	13.56	12.39	0.20	43.39	27.71	0.27

TABLE 17 CUMULATIVE URINARY EXCRETION OF S- AND R-ENANTIOMERS AFTER ADMINISTRATION OF 600 MG OF RACEMIC IBUPROFEN

SUBJECTS	S- mg	R- mg
1	10.46	2.60
2	20.50	3.20
3	26.75	5.40
4	11.04	2.20
5	24.75	4.98
6	10.38	2.50
7	14.93	4.04
8	28.62	6.71
9	10.32	2.13

TABLE 18 PHARMACOKINETIC PARAMETERS IN SUBJECTS RECEIVING 200 MG OF RACEMIC TIAPROFENIC ACID

Subjects	t1/2, h		Vd/F, L		AUC, (mg/L)h		CL/F,mL/min		Xu[2], mg	
	S[1]	R[1]	S	R	S	R	S	R	S	R
1	2.48	2.31	5.17	5.03	69.13	66.23	24.11	25.17	48.33	45.00
2	2.04	2.04	7.36	7.05	39.95	41.50	41.71	39.95	27.93	27.78
3	3.15	3.15	17.24	17.05	26.37	26.51	63.21	62.86	13.94	13.27
4	2.83	2.83	13.07	12.99	28.55	31.41	58.39	53.06	32.82	32.02

[1] S- and R-enantiomers of tiaprofenic acid; [2] cumulative urinary excretion.

TABLE 19 CONCENTRATION OF R- AND S- ENANTIOMERS IN SYNOVIAL FLUID AND PLASMA FOLLOWING SINGLE 200MG DOSES OF RACEMIC TIAPROFENIC ACID

Subjects	Time,h	Concentration, mg/L			
		synovial fluid		plasma	
		S	R	S	R
5	0.75	1.91	1.65	22.22	22.50
	2.00	1.99	1.75	9.64	10.38
6	2.50	1.75	1.62	-	-
	8.00	1.36	1.22	-	-
7	0.25	0.25	0.25	-	-
8	0.50	0.57	0.50	-	-
9	0.25	0.14	0.20	-	-
10	3.00	2.70	2.50	-	-

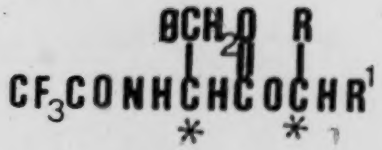
S and R denote the enantiomers of tiaprofenic acid.

TABLE 20 THE ESTIMATED PHARMACOKINETICS PARAMETERS OF d- AND l- ETODOLAC FROM SUBJECTS M AND F FOLLOWING ADMINISTRATION OF THE RACEMIC DRUG

SUBJECTS	M	M	M	F	F	F
ENANTIOMER	d	l	d/l	d	l	d/l
β ug/h.mL	0.111	0.147	0.755	0.081	0.111	0.7297
T1/2 h	6.243	7.714	1.324	8.663	6.930	1.250
AUC 0-24 ug.h/mL	17.924	39.213	0.457	68.405	129.777	0.527
Vd (L)	46.869	16.774	2.794	15.372	6.907	2.225
CL mL/min	52.02	24.66	2.109	12.250	6.222	1.968

FIGURES

10



$$\mathrm{CF_3CONH\overset{\phi CH_2}{\underset{*}{\overset{|}{C}}}H\overset{O}{\overset{\|}{C}}O\overset{R}{\underset{*}{\overset{|}{C}}}HR^1}$$

Figure 1. N-Trifluoroacetyl-S-phenylalanine, (+) alkyl ester.

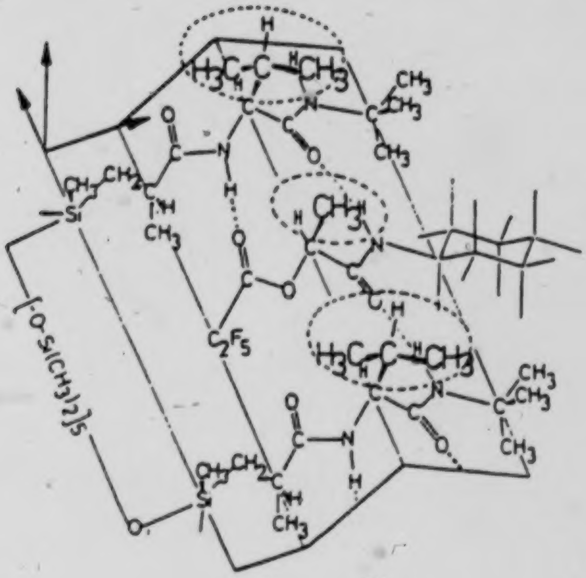
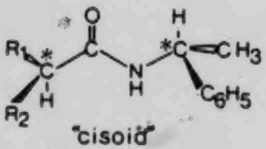
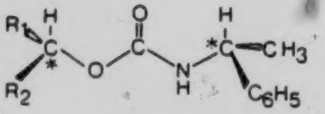

H
H3C-C-CH3
CH3
N
C
CH3
CH3
O
C
N
O
H
CH3
CH2
Si
H
CH3
O
C
O
H
CH3
N
C
O
H
H3C-C-CH3
[-O-Si(CH3)2]5
C2F5
CH3
CH3
N
C
CH3
O
C
N
O
H
CH3
CH2
Si
H
CH3
O

Figure 2. Diastereoisomeric association complexation of N-cyclohexyl-O-pentafluoropropionyl-l-lactamide and CSP of Chirasil-Val. (Frank et al. 1978b).


R1
R2
H
O
C
N
H
H
CH3
C6H5
*

"cisoid"

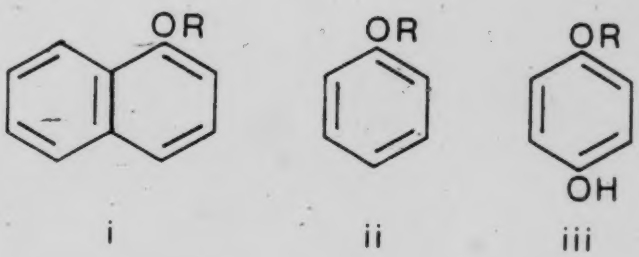
(R,R)- amide


R1
R2
H
O
O
C
N
H
H
CH3
C6H5
*

"cisoid"

(S,S)- carbamate

Figure 3. Solution conformers of diastereoisomeric amides and carbamates. R_1 and R_2 are saturated alkyl groups with R_2 longer than R_1. (Sonnet and Heath 1982).


OR
OR
OR
OH
i
ii
iii

$R = -CH_2CH(OH)CH_2NHCH(CH_3)_2$

Figure 4. Structural formulae of propranolol (i), pre-prenalterol (ii) and prenalterol (iii).

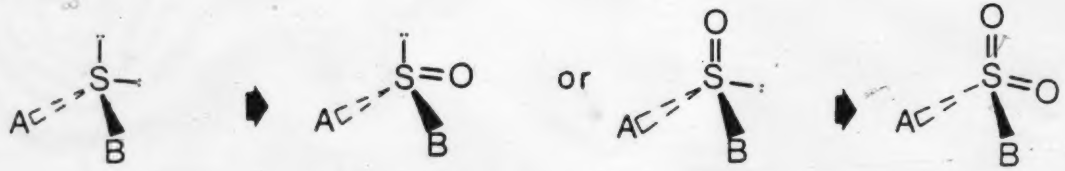


Figure 5. Scheme for microbiological oxidation of sulfides (Smith and Rosazza, 1975)

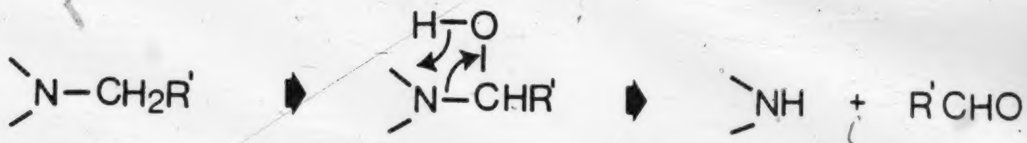

N−CH2R'
H−O
N−CHR'
NH
+
R'CHO

Figure 6. Scheme for N-dealkylation via carbinolamine formation.

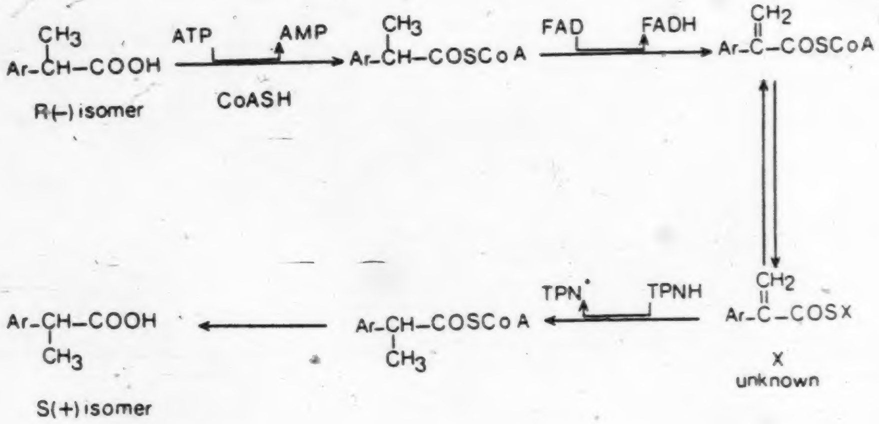
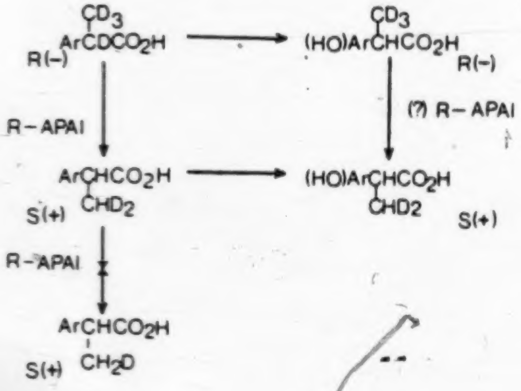
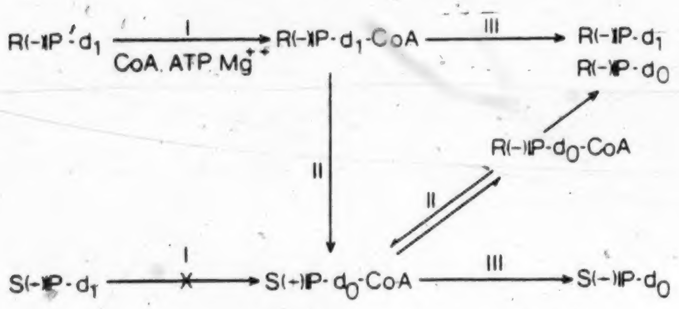

CH3
Ar-CH-COOH
R(-) isomer
ATP
AMP
CoASH
CH3
Ar-CH-COSCoA
FAD
FADH
CH2
Ar-C-COSCoA
CH2
Ar-C-COSX
X
unknown
TPN+
TPNH
Ar-CH-COSCoA
CH3
Ar-CH-COOH
CH3
S(+) isomer

Figure 7. Epimerization mechanism of R-APAI (R-Arylpropionic acid isomerase). (Wechter et al 1974).



CD3
ArCDCO2H
R(−)
(HO)ArCHCO2H
R(−)
R−APAI
(?) R−APAI
ArCHCO2H
CHD2
S(+)
(HO)ArCHCO2H
CHD2
S(+)
R−APAI
ArCHCO2H
CH2D
S(+)

Figure 8. Proposed pathway for the inversion of deuterated arylpropionic acids in humans. (Wechter et al 1974).


R(-)IP-d1
I
CoA, ATP, Mg++
R(-)IP-d1-CoA
III
R(-)IP-d1
R(-)IP-d0
II
R(-)IP-d0-CoA
II
S(-)IP-d1
I
S(+)IP-d0-CoA
III
S(-)IP-d0

IP - Ibuprofen

I - R(-) Ibuprofenyl CoA synthetase

II - R(-) Ibuprofenyl CoA racemase

III - R(-) Ibuprofenyl CoA hydrolase

Figure 9. Proposed mechanism for stereoselective isomerization of R (-) ibuprofen to S (+) ibuprofen. (Nakamura et al 1981)

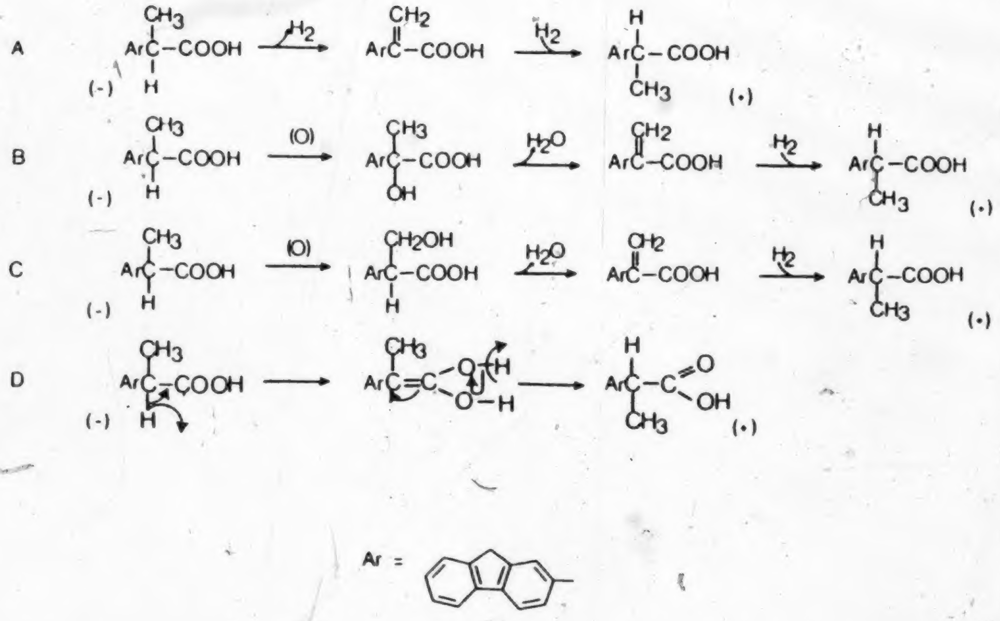

A
B
C
D
ArC−COOH
CH3
CH2
H
OH
CH2OH
H2
(O)
H2O
(-)
(+)
Ar =

Figure 10. Proposed inversion mechanisms for cicloprofen (Lan et al 1976).

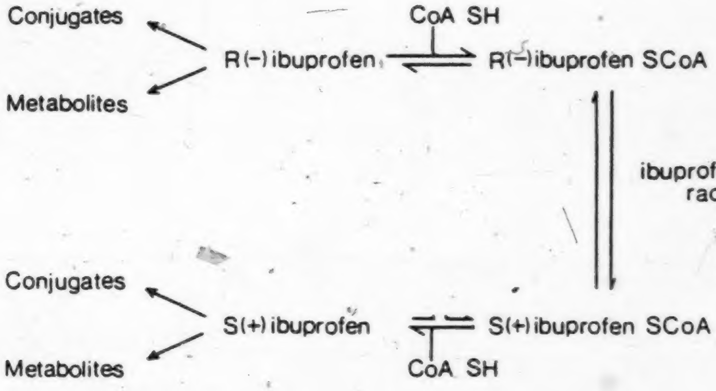

Conjugates
Metabolites
R(−) ibuprofen
CoA SH
R(−)ibuprofen SCoA
ibuprofen SCoA racemase
Conjugates
Metabolites
S(+) ibuprofen
S(+) ibuprofen SCoA
CoA SH

Figure 11. Proposed mechanism of the inversion reaction of R (-) to S (+)-ibuprofen. (Lee et al 1985).

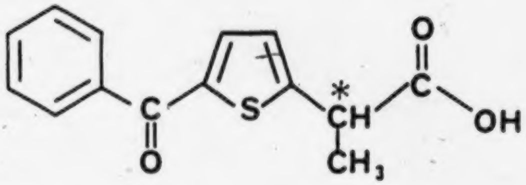


Figure 12. Tiaprofenic Acid.

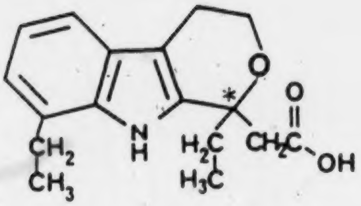

O
*
O
N
H
CH_2
CH_3
H_2C
H_3C
CH_2
C
OH

Figure 13. Etodolac.

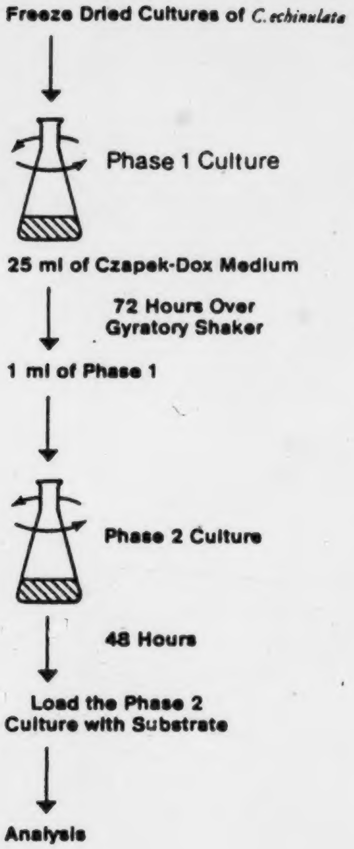
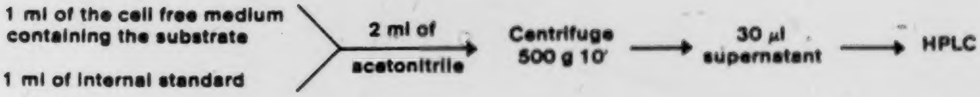

Freeze Dried Cultures of *C. echinulata*
Phase 1 Culture
25 ml of Czapek-Dox Medium
72 Hours Over Gyratory Shaker
1 ml of Phase 1
Phase 2 Culture
48 Hours
Load the Phase 2 Culture with Substrate
Analysis

Figure 14. A typical methodology for incubation and sample loading of microorganisms for metabolic studies.


1 ml of the cell free medium containing the substrate
1 ml of internal standard
2 ml of
acetonitrile
Centrifuge
500 g 10'
30 μl
supernatant
HPLC

HPLC Conditions

Column System: Waters μ·Bondapak C_{18} (reverse phase)
Mobile Phase: Methanol: Water: ACOH [50:48:2]
Flow Rate: 2 ml/min at Room Temperature [20°C]
Wavelength 272 nm.

Figure 15. Protocol for HPLC analysis of cephalosporanic acid and its sulphoxide.

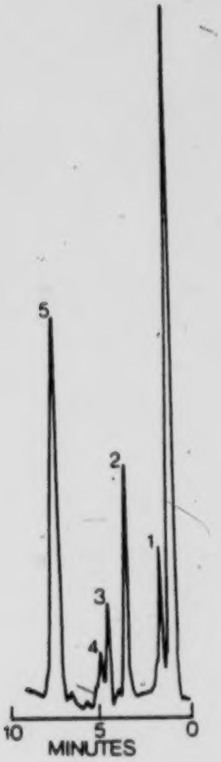


Figure 16. Liquid chromatogram of *C. echinulata* cultures incubated with 7-[(phenoxy)acetamido]desacetylcephalospranic acid, methyl esters (CS). Key: 1=CSO; 2=CS; 3 & $ 4=endogenous components; 5=internal standard (ethyl ester of CS).

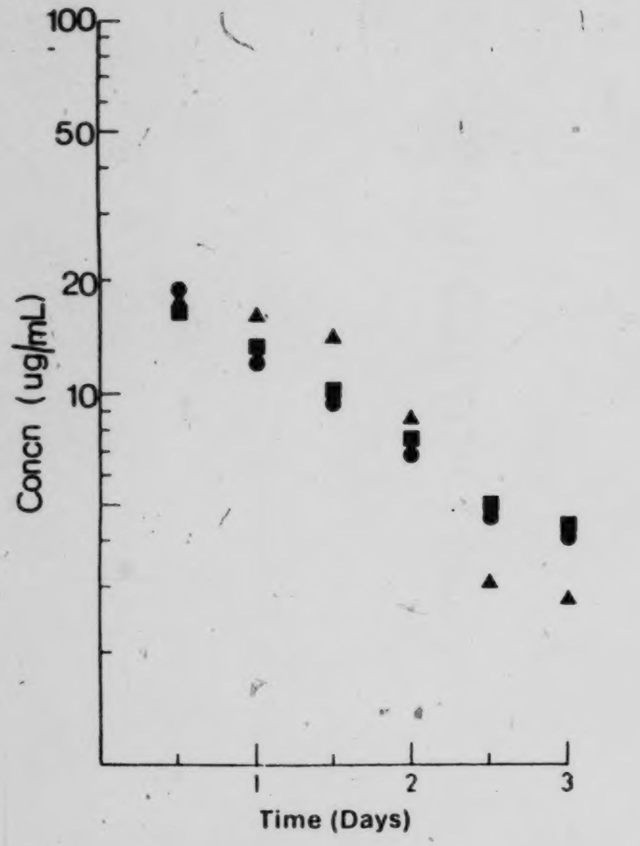


Figure 17A. Rate of CS disappearance at CS concentration of 25 ug/mL.

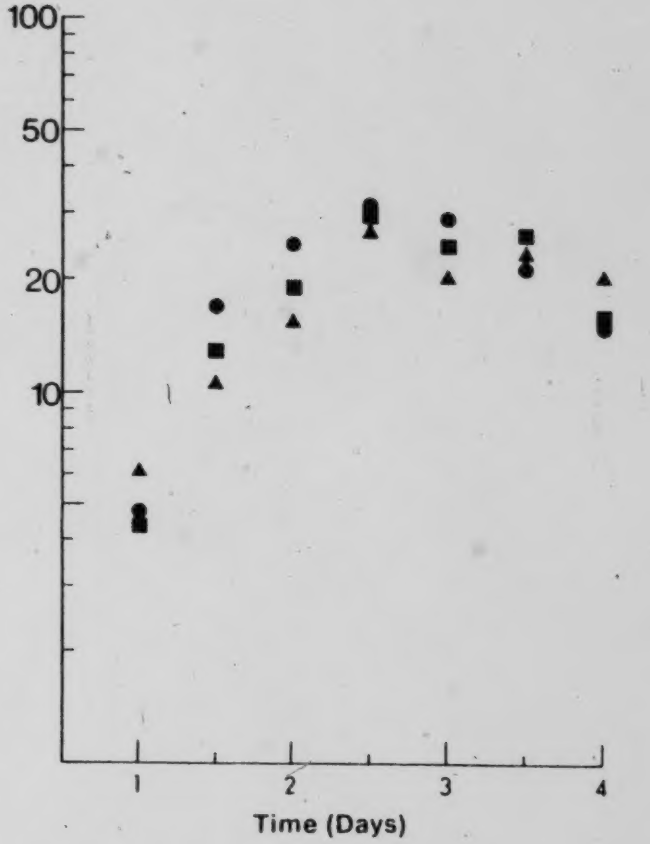


Figure 17B. Rate of CSO formation at CS concentration of 25 ug/mL.

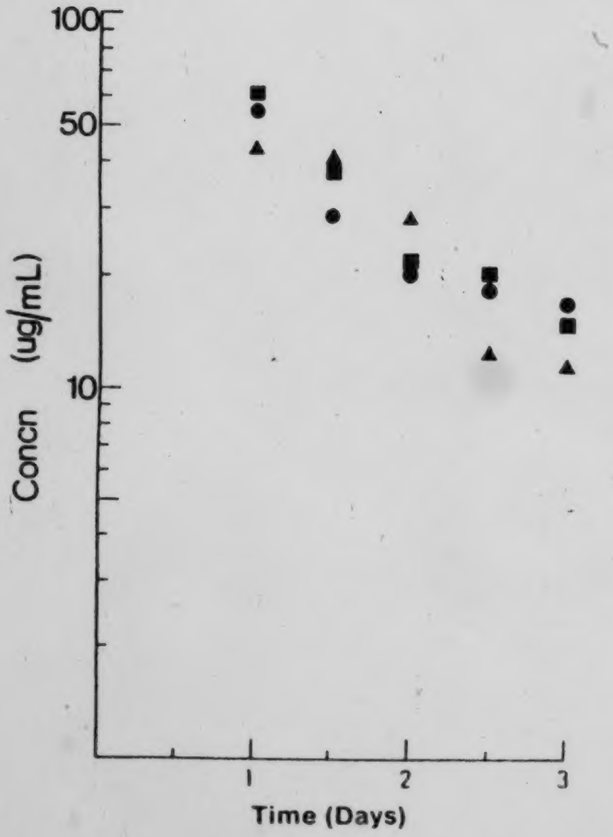


Figure 18A. Rate of CS disappearance at CS concentration of 100 ug/mL.

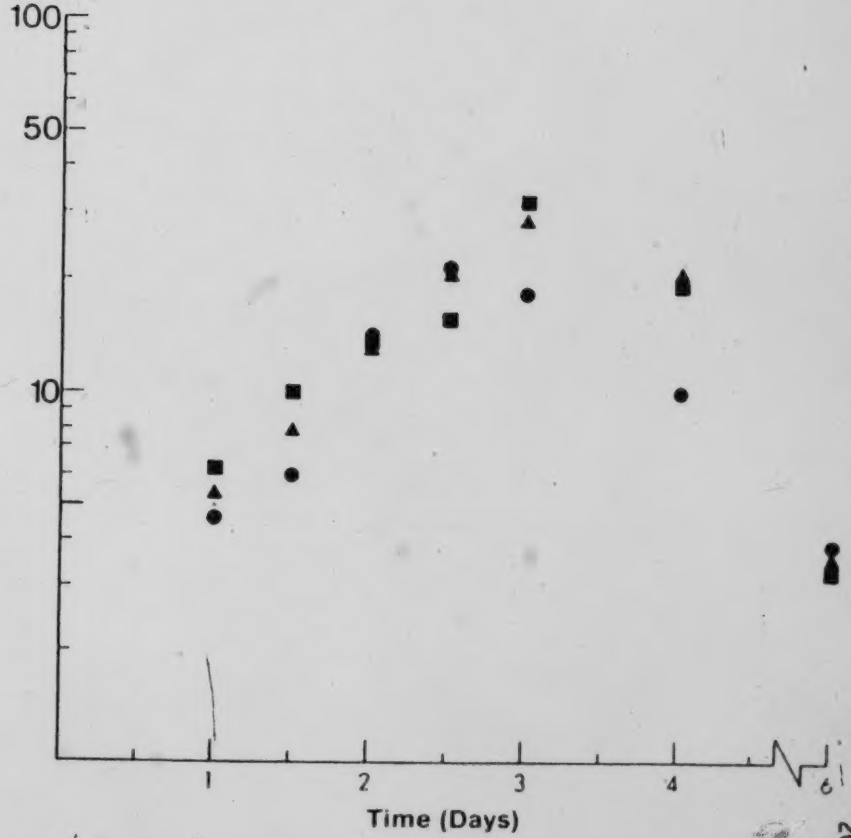


Figure 18B. Rate of CSO formation at CS concentration of 100 ug/mL.

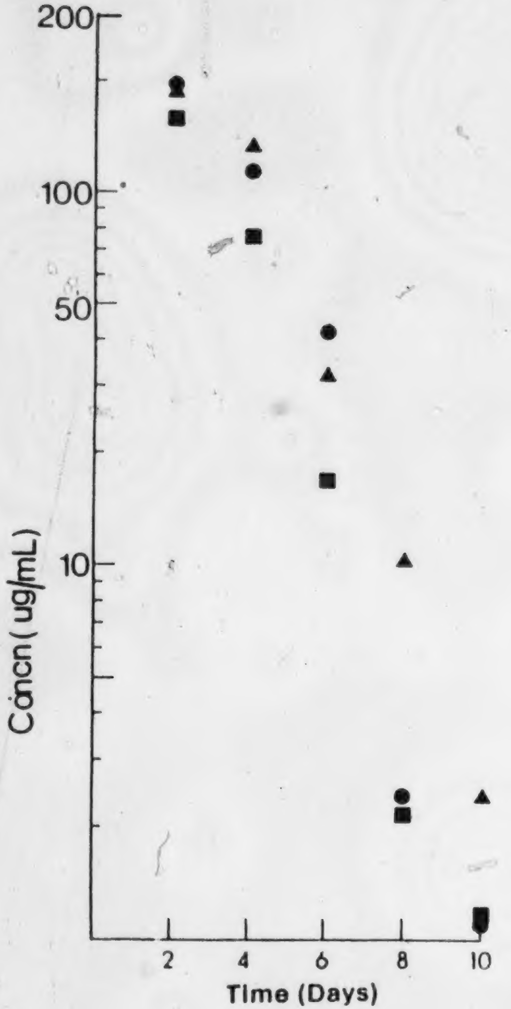


Figure 19A. Rate of CS disappearance at CS concentration of 200 ug/mL.

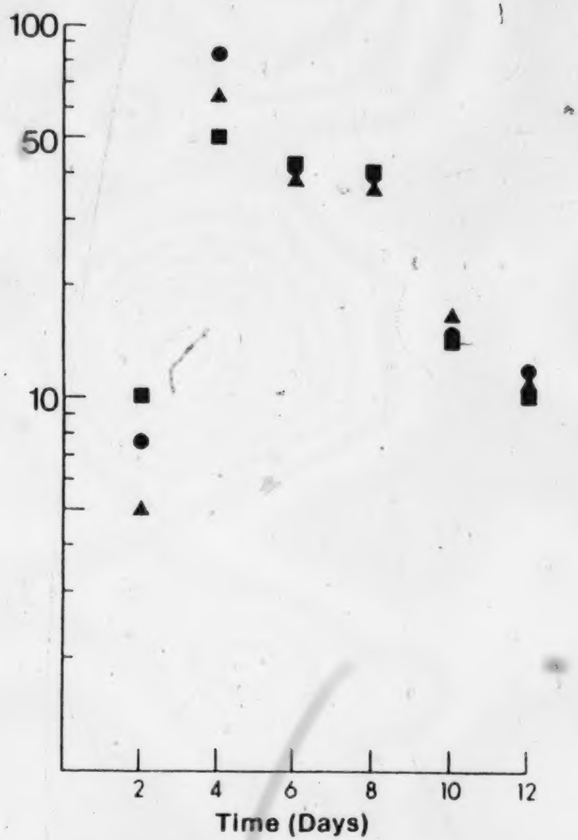


Figure 19B. Rate of CSO formation at CS concentration of 200 ug/mL.

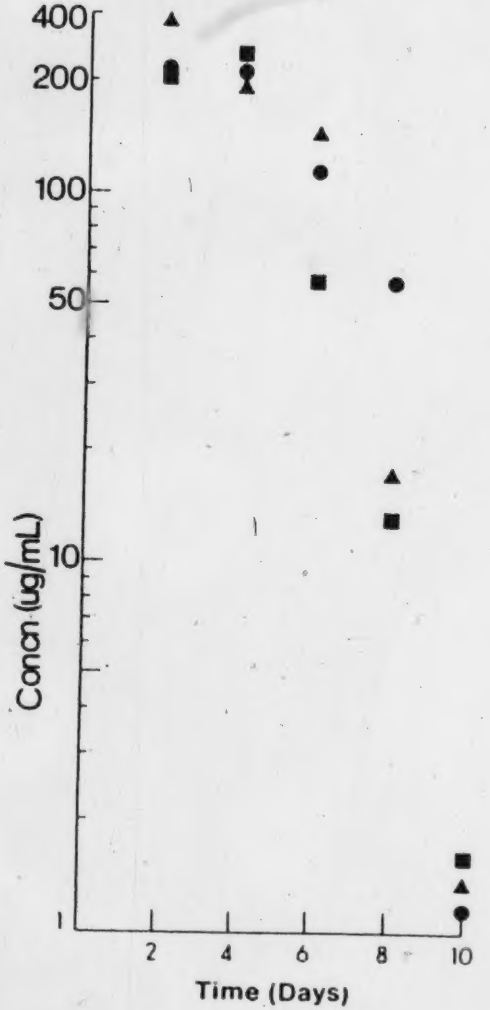


Figure 20A. Rate of CS disappearance at CS concentration of 400 ug/mL.

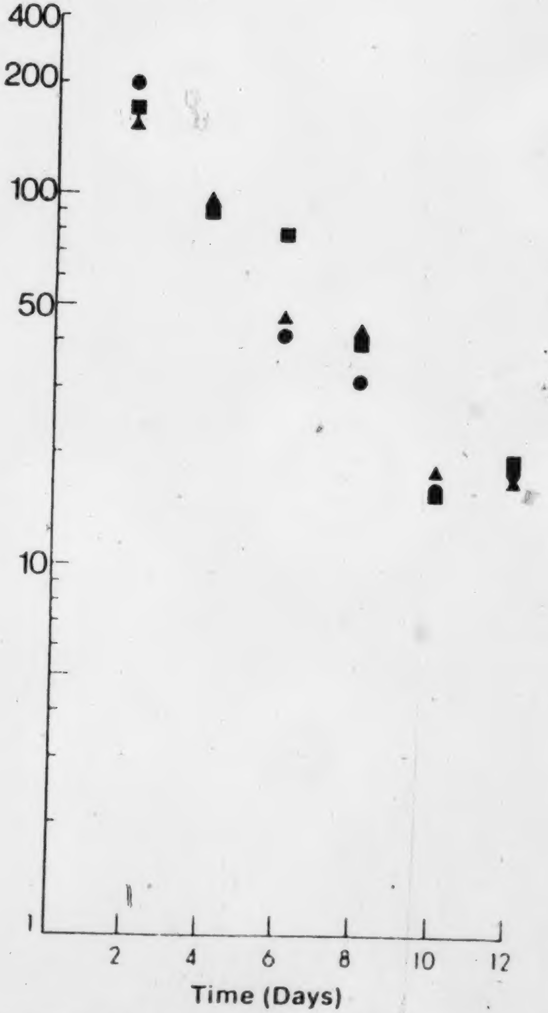


Figure 20B. Rate of CSO formation at CS concentration of 400 ug/mL.

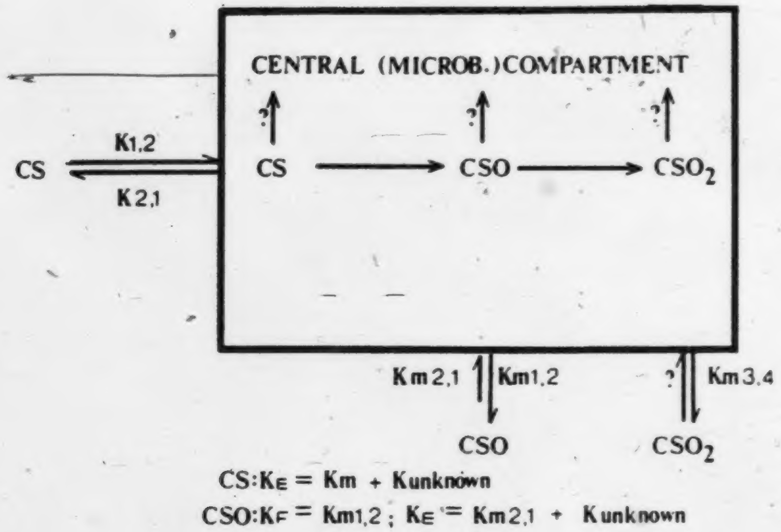

CENTRAL (MICROB.)COMPARTMENT
?
?
?
CS
K1,2
K2,1
CS
CSO
CSO2
Km2,1
Km1,2
?
Km3,4
CSO
CSO2
CS:KE = Km + Kunknown
CSO:KF = Km1,2 ; KE = Km2,1 + Kunknown

Figure 21· Proposed kinetic model for *C. echinulata*-mediated metabolism of CS.
K1,2 and K2,1 are equilibrium rate constants for CS within the culture medium and within the microorganism.
Km1,2 is the metabolic rate constant for CSO formation.
Km3,4 is the metabolic rate constant for CSO_2 formation and is approximately equal to Km2,1.

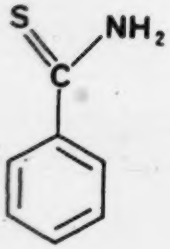


Figure 22 Thiobenzamide

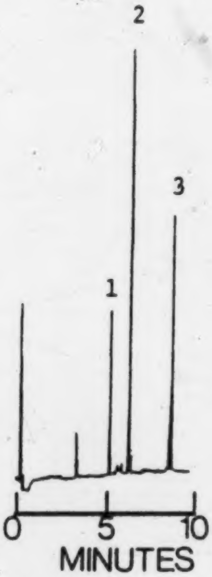


Figure 23. Gas chromatogram of derivatized extract of incubate containing preprenalterol (1, 5.09 min), prenalterol (2, 6.14 min) and propranolol (3, 8.34 min).

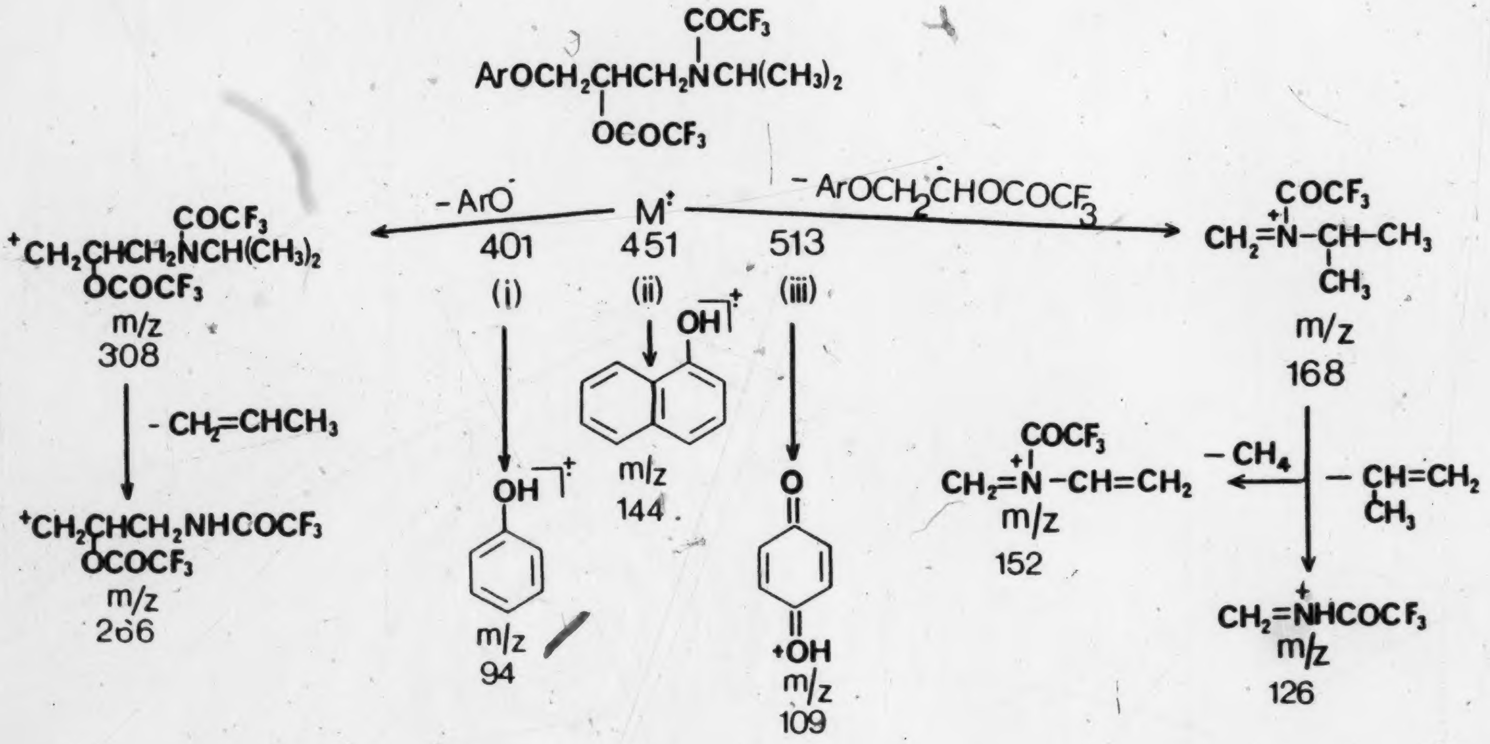

COCF3
ArOCH2CHCH2NCH(CH3)2
OCOCF3
- ArO
M
401
451
513
(i)
(ii)
(iii)
- ArOCH2CHOCOCF3
COCF3
CH2=N-CH-CH3
CH3
m/z
168
COCF3
+CH2CHCH2NCH(CH3)2
OCOCF3
m/z
308
- CH2=CHCH3
+CH2CHCH2NHCOCF3
OCOCF3
m/z
266
OH
m/z
94
OH
m/z
144
O
+OH
m/z
109
- CH4
- CH=CH2
CH3
COCF3
CH2=N-CH=CH2
m/z
152
CH2=NHCOCF3
m/z
126

Figure 24. Proposed structures for major mass spectral fragments of N,O-bis-(trifluoroacetylated)-preprenalterol (i), N,O-bis-(trifluoroacetylated) propranolol (ii), and N,O,O-tris-(trifluoroacetylated) prenalterol (iii).

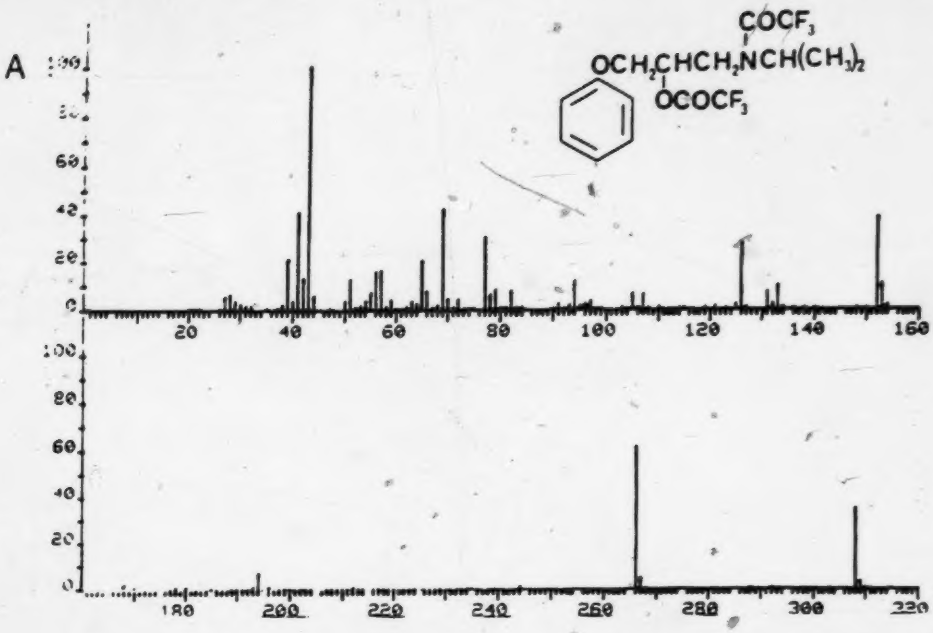


Figure 25A. MS-line diagram of N,O-bis(trifluoroacetyl)preprenalterol.

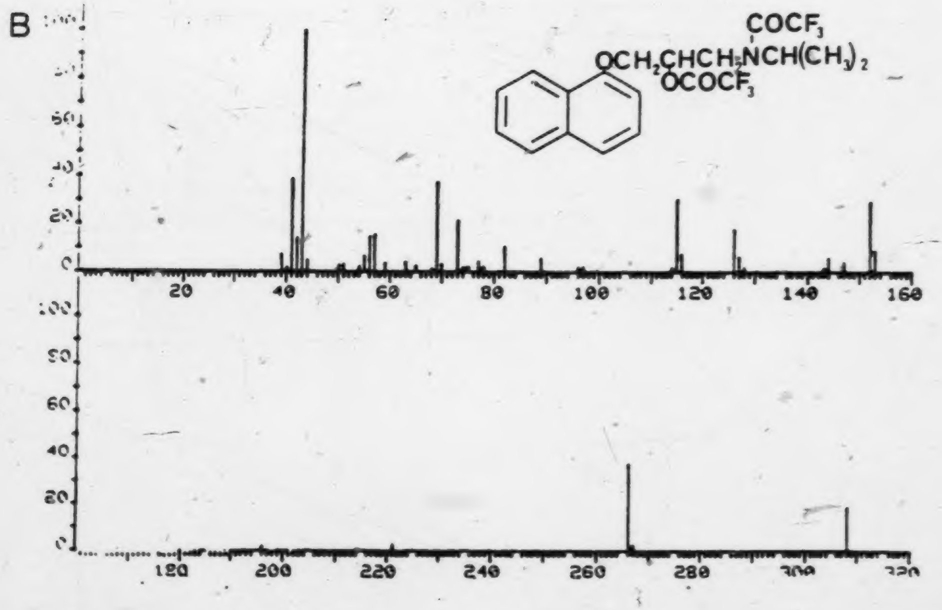


Figure 25B. MS-line diagram of N,O-bis(trifluoroacetyl)propranolol.

C

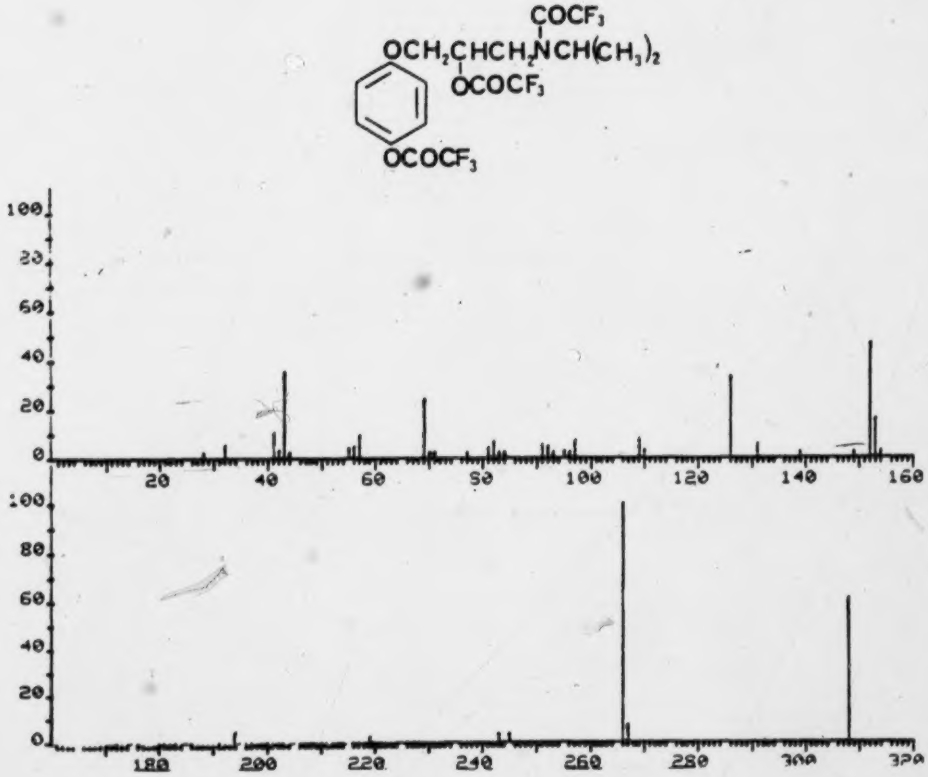


Figure 25C. MS-line diagram of N,O,O-tris(trifluoroacetylated)prenalterol.

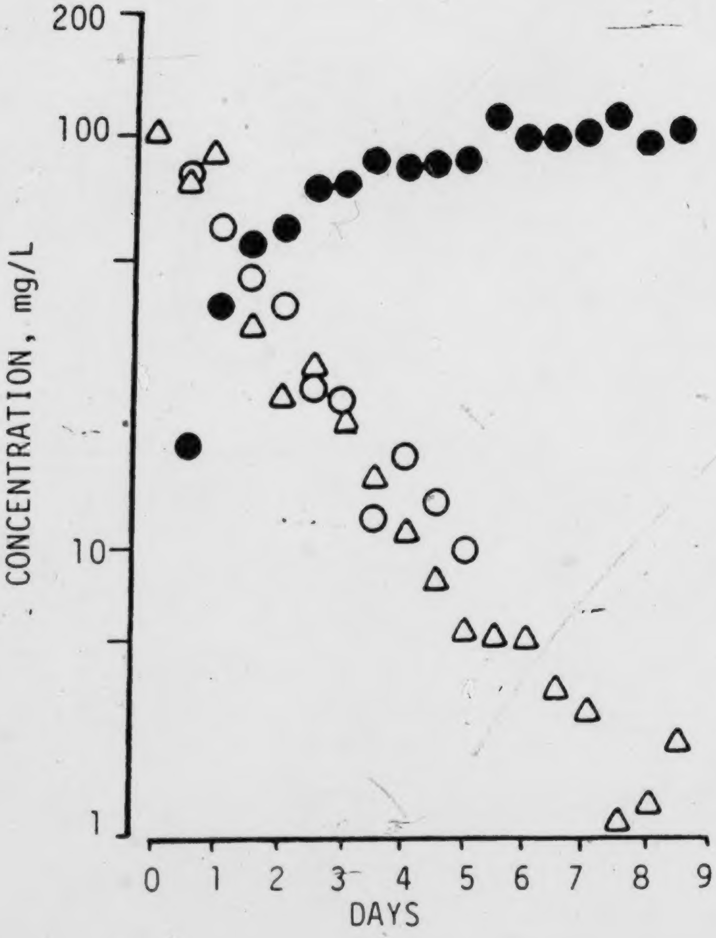


Figure 26. Concentration-time curves for preprenalterol (△) and cumulative concentration of prenalterol (●), obtained after initial substrate concentration of 100 mg/L.
(○) represents the concentration of prenalterol remaining to be formed.

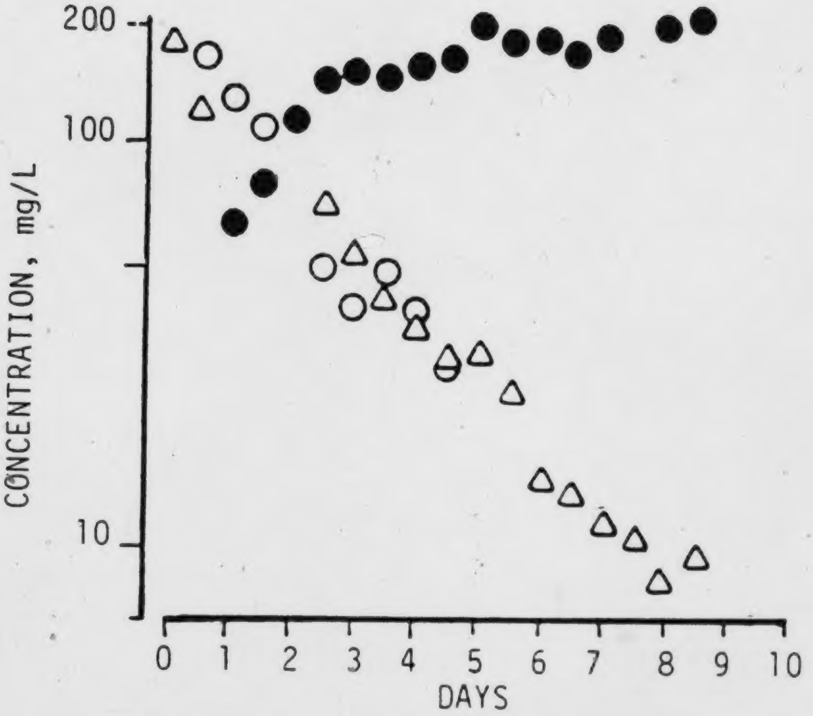


Figure 27. Concentration-time curves for preprenalterol (△) and cumulative concentration of prenalterol (●), obtained from initial substrate concentration of 200 mg/L.
(○) represents the concentration of prenalterol remaining to be formed.

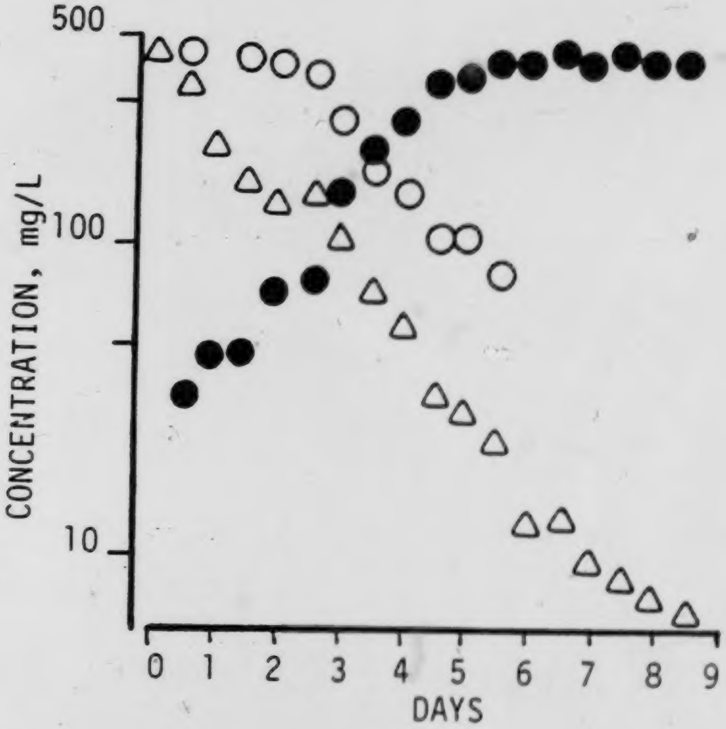


Figure 28. Concentration-time curves for preprenalterol (△) and cumulative concentration of prenalterol (●), obtained from initial substrate concentration of 400 mg/L.

(○) represents the concentration of prenalterol remaining to be formed.

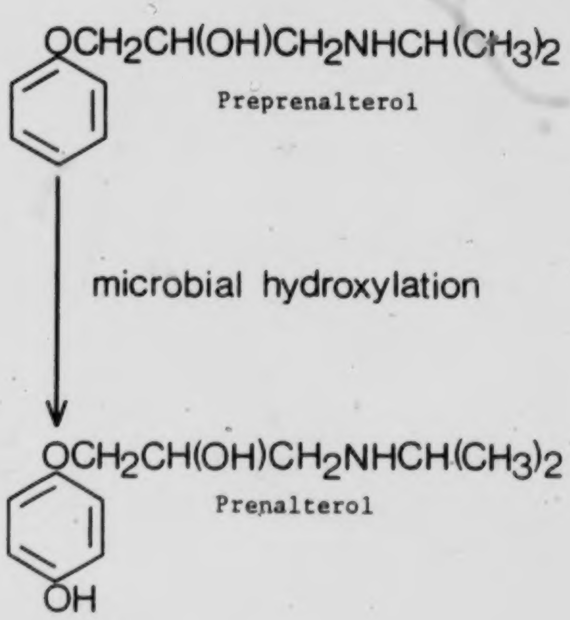

OCH2CH(OH)CH2NHCH(CH3)2
Preprenalterol
microbial hydroxylation
OCH2CH(OH)CH2NHCH(CH3)2
Prenalterol
OH

Figure 29. *C. echinulata*-mediated *para*-hydroxylation.

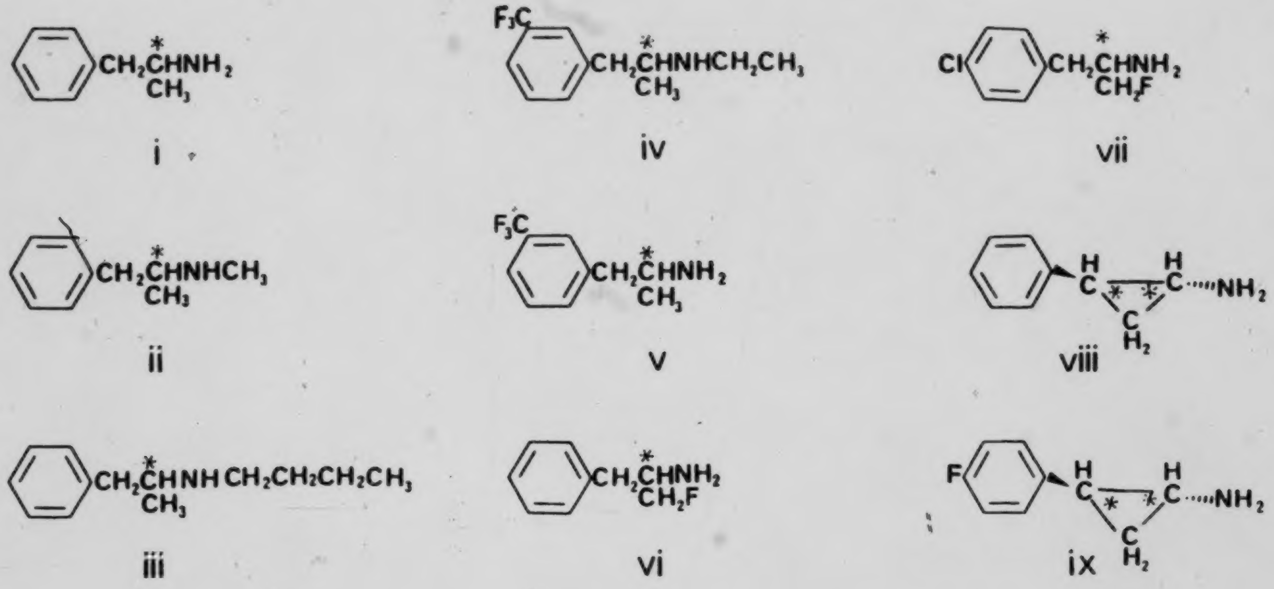
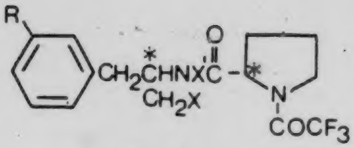


Figure 30. Structures of amphetamine analogs resolved after derivatization with N-trifluoroacetyl-L-prolyl chloride. i = amphetamine, ii = methylamphetamine, iii = N-n-butylamphetamine, iv = fenfluramine, v = norfenfluramine, vi = fluoroamphetamine, vii = p-chloro-fluoroamphetamine, viii = tranylcypromine, ix= p-fluorotranylcypromine; * denotes an asymmetric center.



R	X	X'	
H	H	H	i
H	H	CH_3	ii
H	F	H	iii
CF_3	H	C_2H_5	iv
CF_3	H	H	v

Figure 31. Structures of derivatized N-TPC diastereomers of (i) amphetamine, (ii) methylamphetamine, (iii) fluoroamphetamine, (iv) fenfluramine and (v) norfenfluramine; * denotes an asymmetric center.

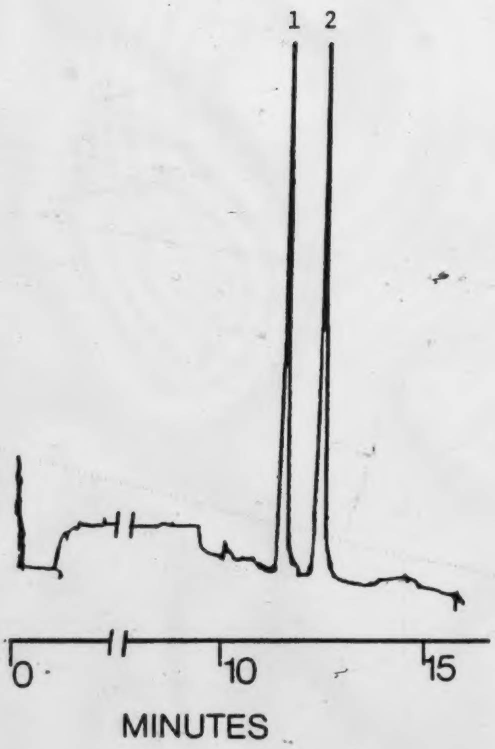


Figure 32. Gas chromatogram of R,S-amphetamine-N-TPC diastereomer. Key: 1 = R(-)-amphetamine-N-TPC, 2 = S(+)-amphetamine-N-TPC.

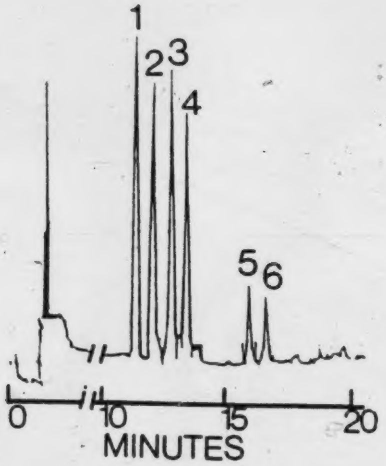


Figure 33. Resolution of diastereomeric derivatives of amphetamine analogs in a single chromatographic run. Key: 1 = R(-)-amphetamine-N-TPC, 2 = S(+)-amphetamine-N-TPC, 3 = S(-)-fluoroamphetamine-N-TPC, 4 = R(+)-fluoroamphetamine-N-TPC, 5 = R(-)-methylamphetamine-N-TPC and 6 = S(+)-methylamphetamine-N-TPC.

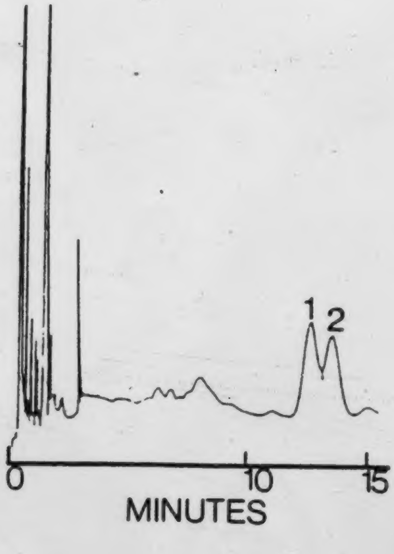


Figure 34. GC trace of resolution of R,S-methylamphetamine-N-TPC on OV-101 packed column. Key: 1 = R(-)-methylamphetamine-N-TPC and 2 = S(+)-methylamphetamine-N-TPC.

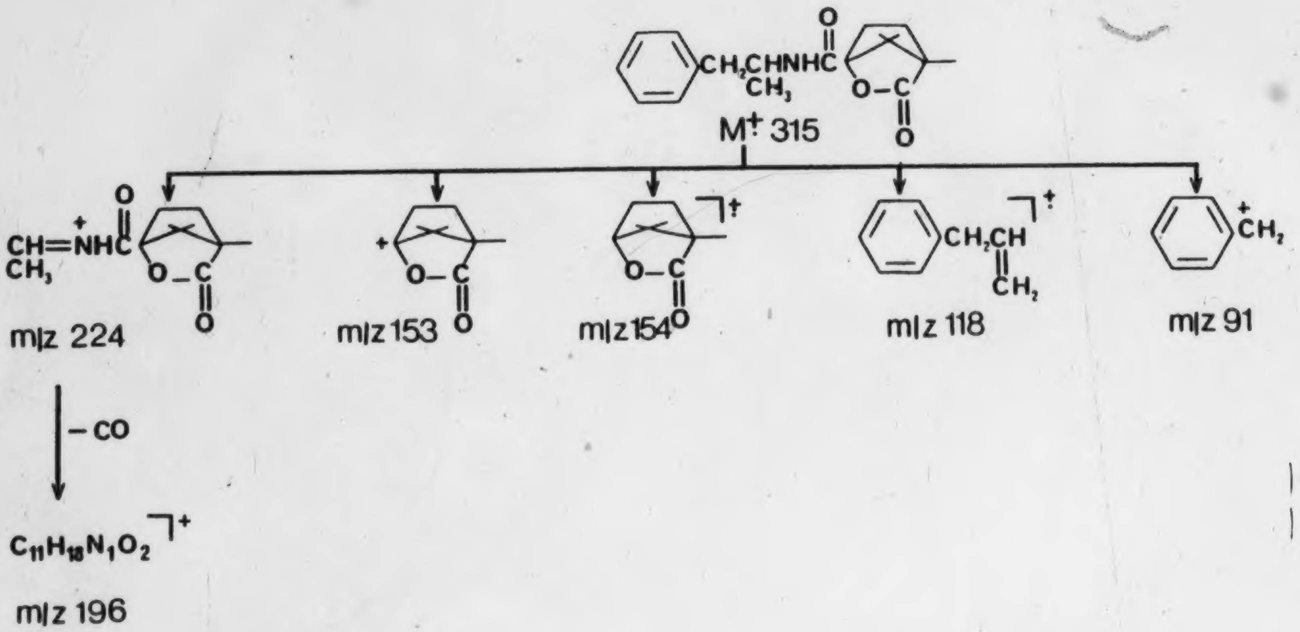

CH₂CHNHC
CH₃
M⁺˙ 315
CH=N⁺HC
CH₃
m/z 224
m/z 153
m/z 154
CH₂CH
CH₂
m/z 118
CH₂
m/z 91
-CO
C₁₁H₁₈N₁O₂
m/z 196

Figure 35. Proposed structures for the MS fragments of R,S-amphetamine-(-)-camphanic acid chloride derivative.

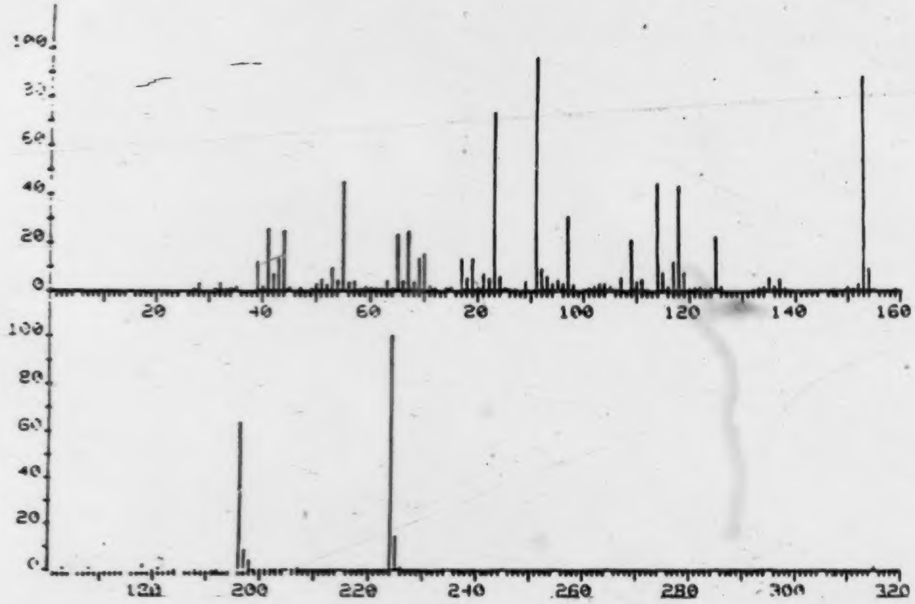


Figure 36. MS-line diagram of R,S-amphetamine-(-)-camphanic acid chloride derivative.

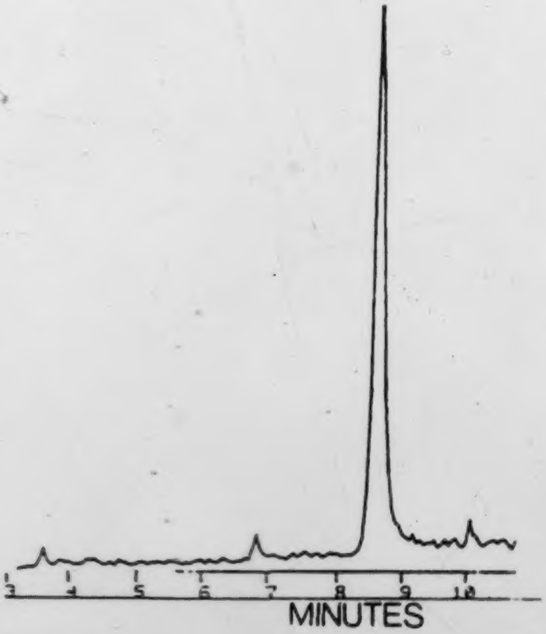


Figure 37. Total ion GC-MS trace of R,S-amphetamine-(-)-camphanic acid chloride derivative.

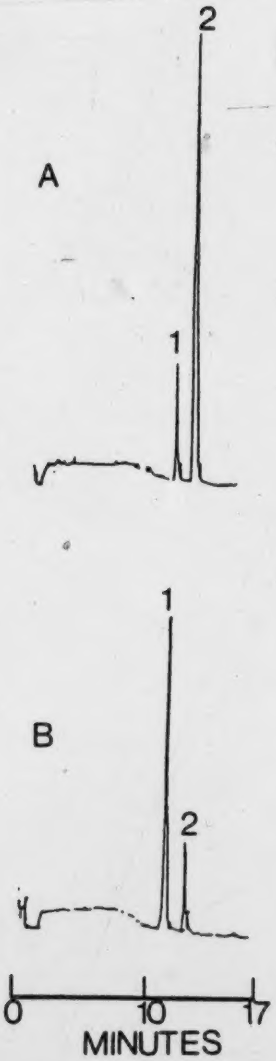


Figure 38. Gas chromatograms of N-TPC derivatized, enriched isomers of (A) S(+)-amphetamine and (B) R(-)-amphetamine. Key: 1 = R(-)-amphetamine-N-TPC and 2 = S(+)-amphetamine-N-TPC.

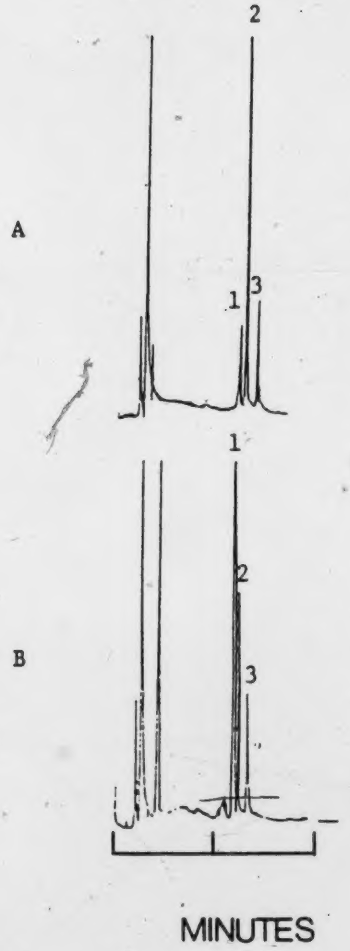


Figure 39. Gas chromatograms of N-TPC derivatized, enriched isomers of (A) R(+)-fluoroamphetamine and (B) S(-)-fluoroamphetamine.
Key: 1 = S(-)-fluoroamphetamine-N-TPC, 2 = R(+)-fluoroamphetamine-N-TPC and 3 = reagent peak.

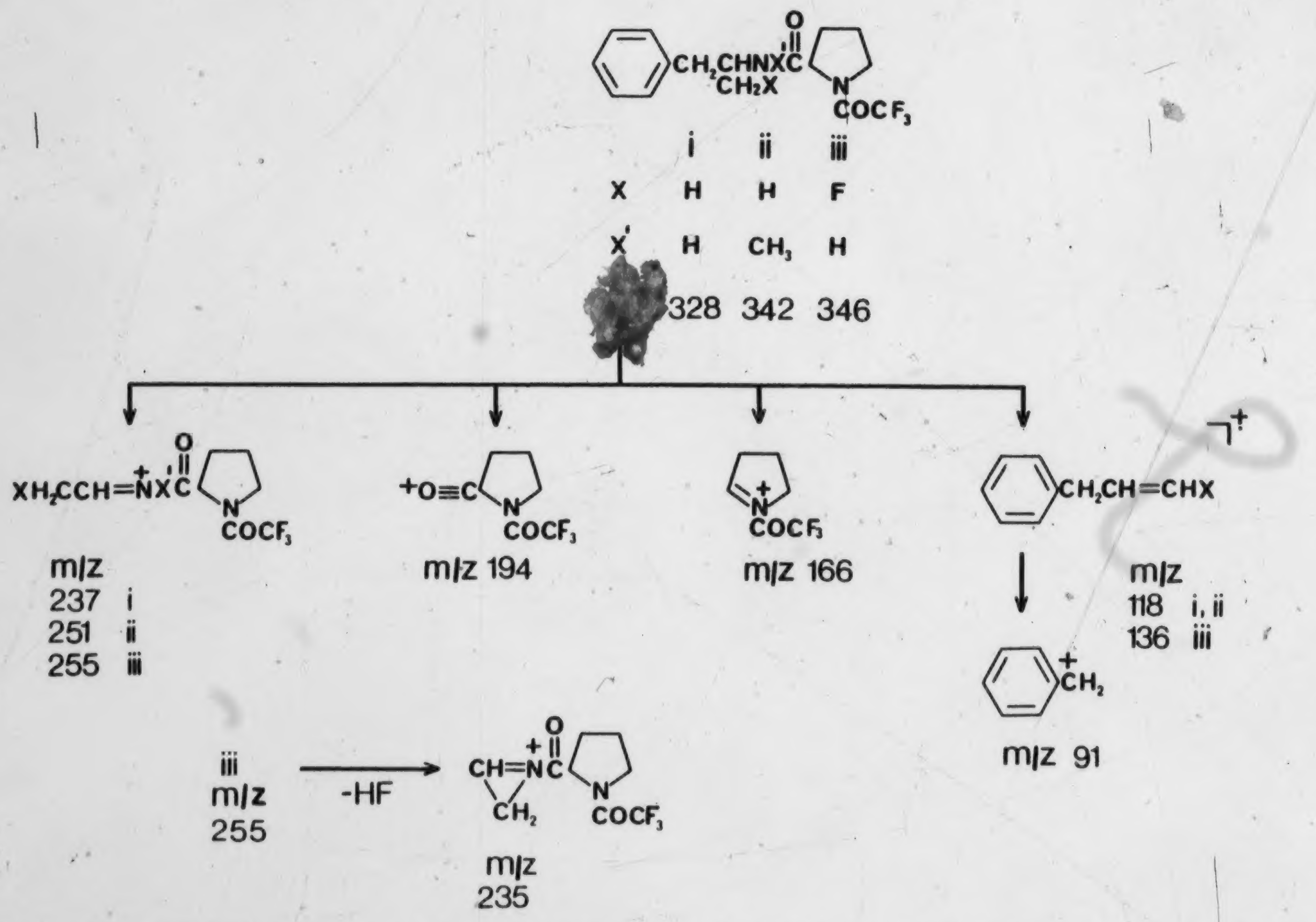

CH2CHNX'C(O)
CH2X
COCF3
i ii iii
X H H F
X' H CH3 H
328 342 346
XH2CCH=N+X'C(O)
COCF3
m/z
237 i
251 ii
255 iii
+O≡C
COCF3
m/z 194
N+
COCF3
m/z 166
CH2CH=CHX
m/z
118 i, ii
136 iii
+CH2
m/z 91
iii
m/z
255
-HF
CH=N+C(O)
CH2
COCF3
m/z
235

Figure 40. Proposed structures for MS fragments of diastereomeric derivatives of (i) amphetamine, (ii) methylamphetamine and (iii) fluoroamphetamine.

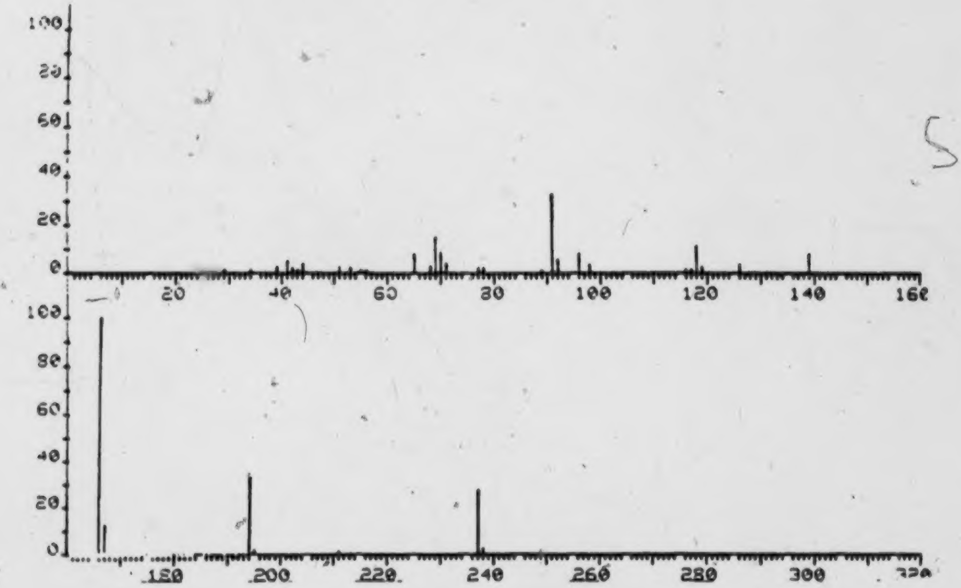


Figure 41. MS-line diagram of R,S-amphetamine-N-TPC diastereomer.

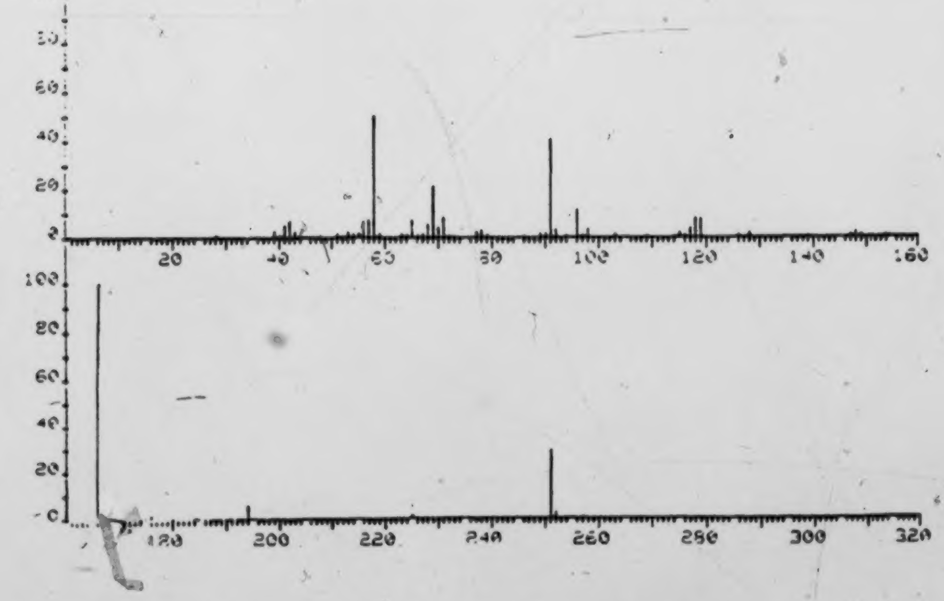


Figure 42. MS-line diagram of R,S-methylamphetamine-N-TPC diastereomer.

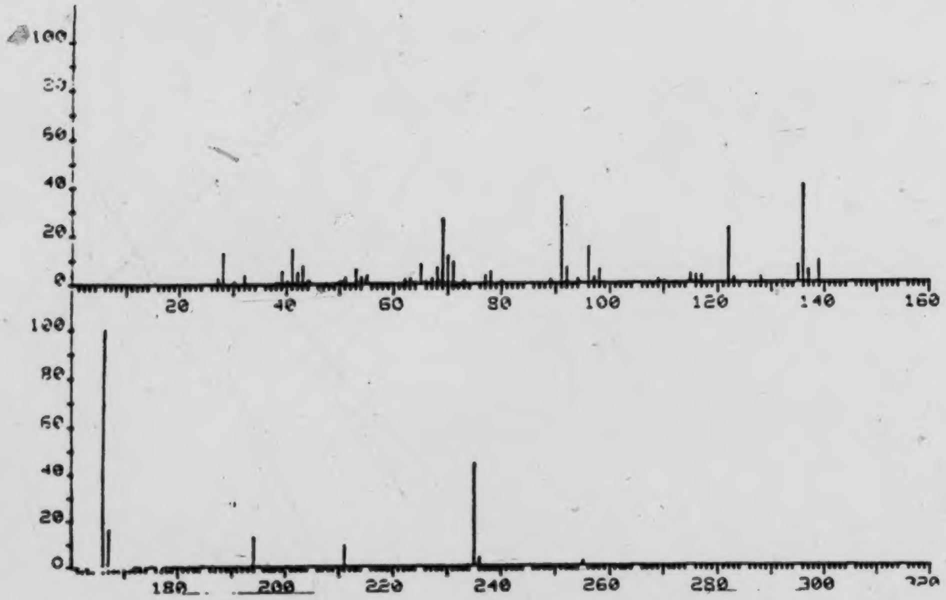


Figure 43. MS-line diagram of R,S-fluoroamphetamine-N-TPC diastereomer.

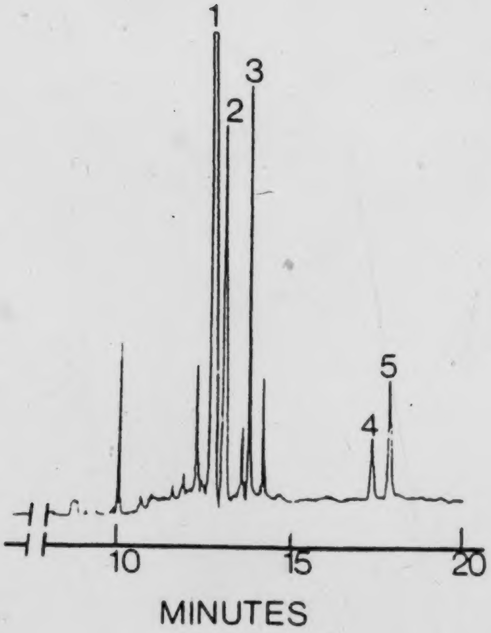


Figure 44. Gas chromatogram of N-TPC derivatized extract of R,S-fenfluramine - dosed rat (brain) sample.
Key: 1 = R(-)-norfenfluramine-N-TPC, 2 = S(+)-norfenfluramine-N-TPC, 3 = R(+)-fluoroamphetamine (internal standard)-N-TPC, 4 = R(-)-fenfluramine-N-TPC and 5 = S(+)-fenfluramine-N-TPC.

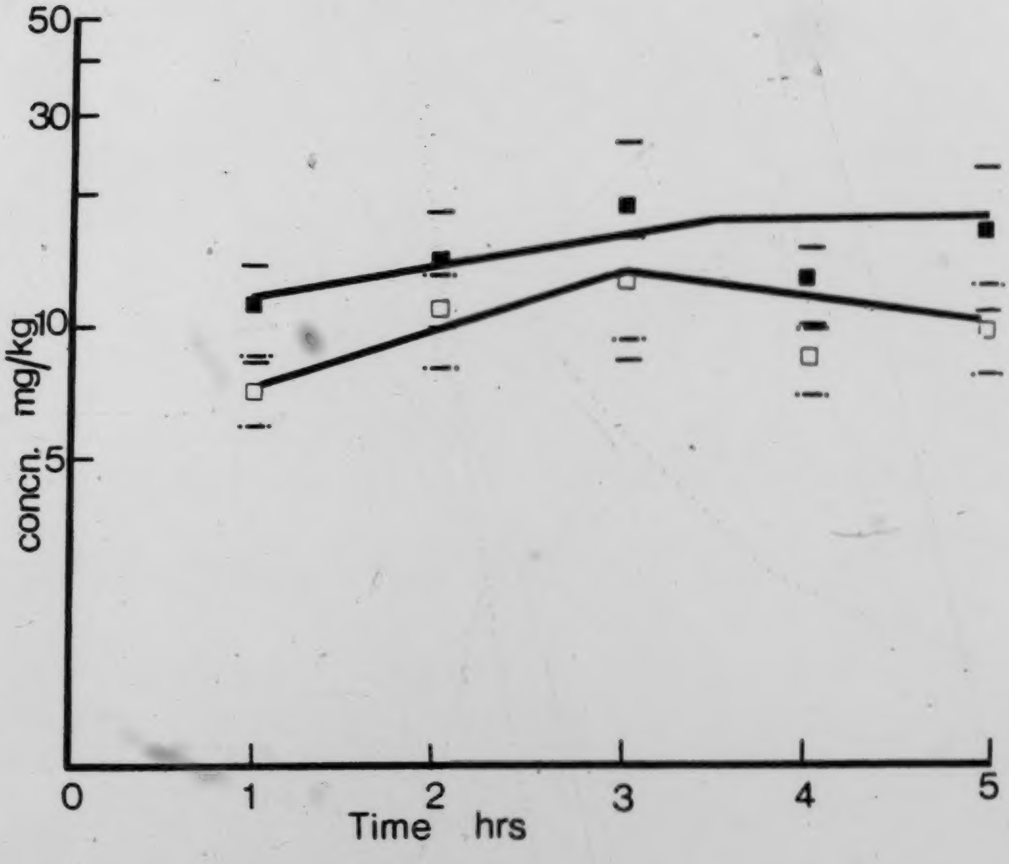


Figure 45. Rat brain levels of S(+) [■] and R(-) [□] fenfluramine after intraperitoneal administration of racemic fenfluramine (40 mg/kg).

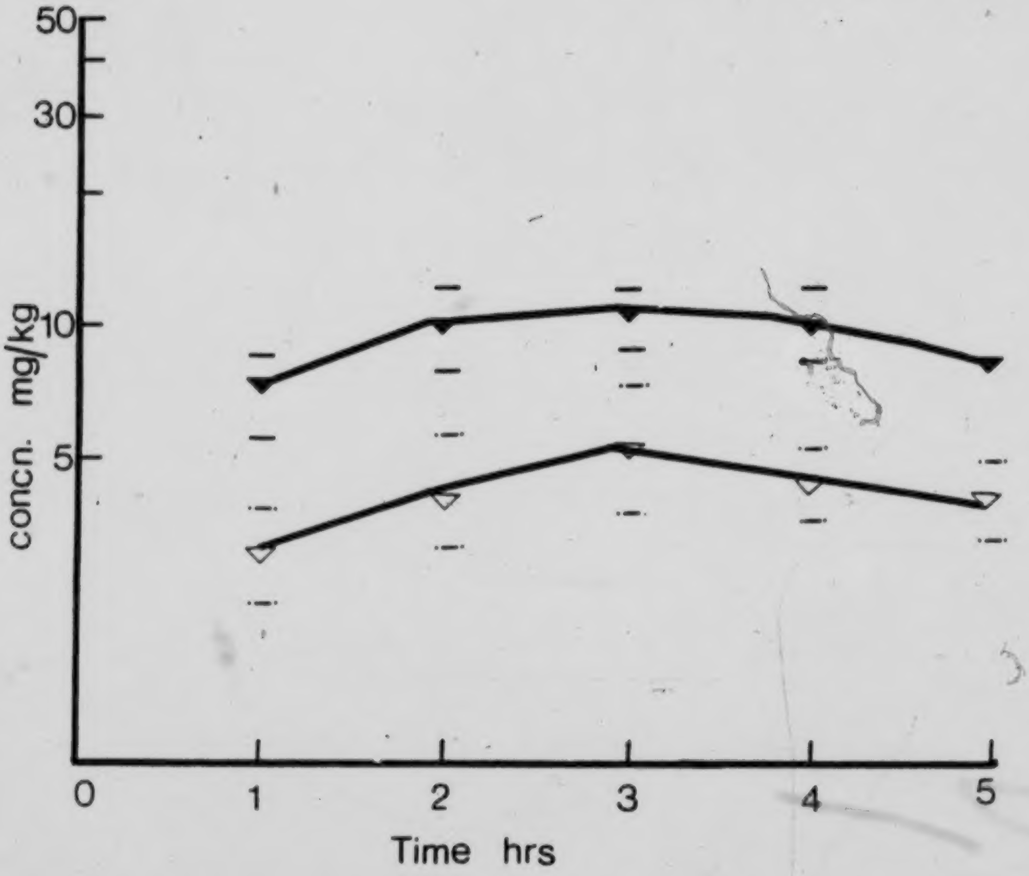


Figure 46. Rat brain levels of R(-) [▼] and S(+) [▽] norfenfluramine after intraperitoneal administration of racemic fenfluramine (40 mg/kg).

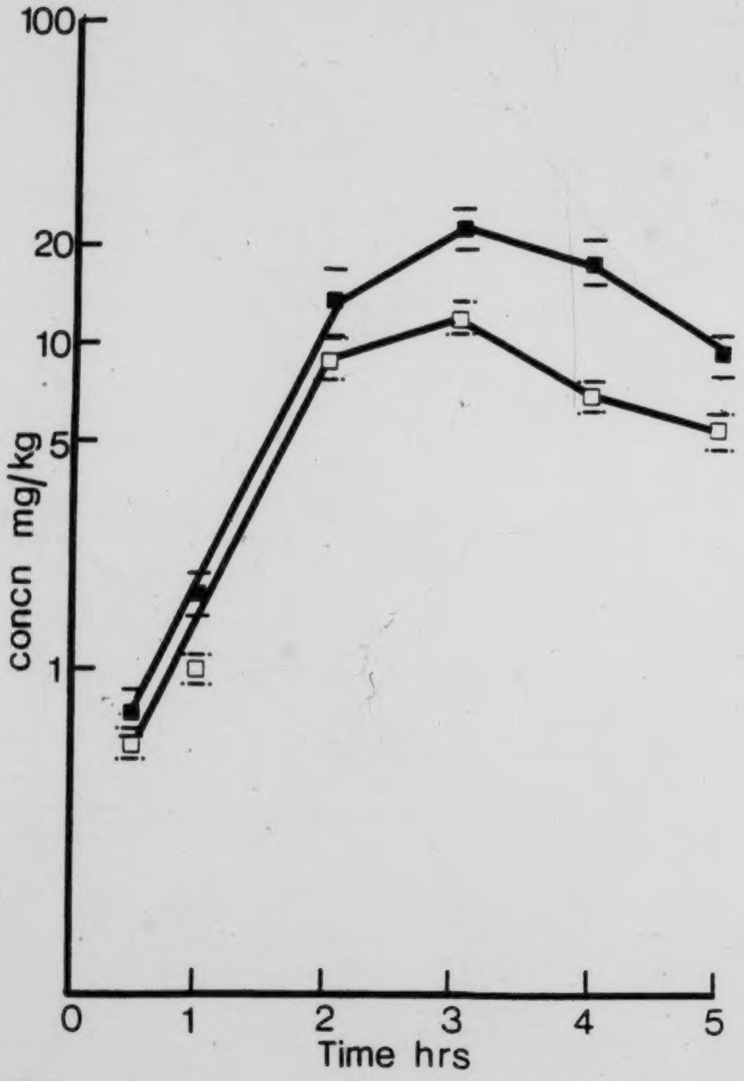


Figure 47. Rat liver levels of S(+) [■] and R(-) [□] fenfluramine after intraperitoneal administration of racemic fenfluramine (40 mg/kg).

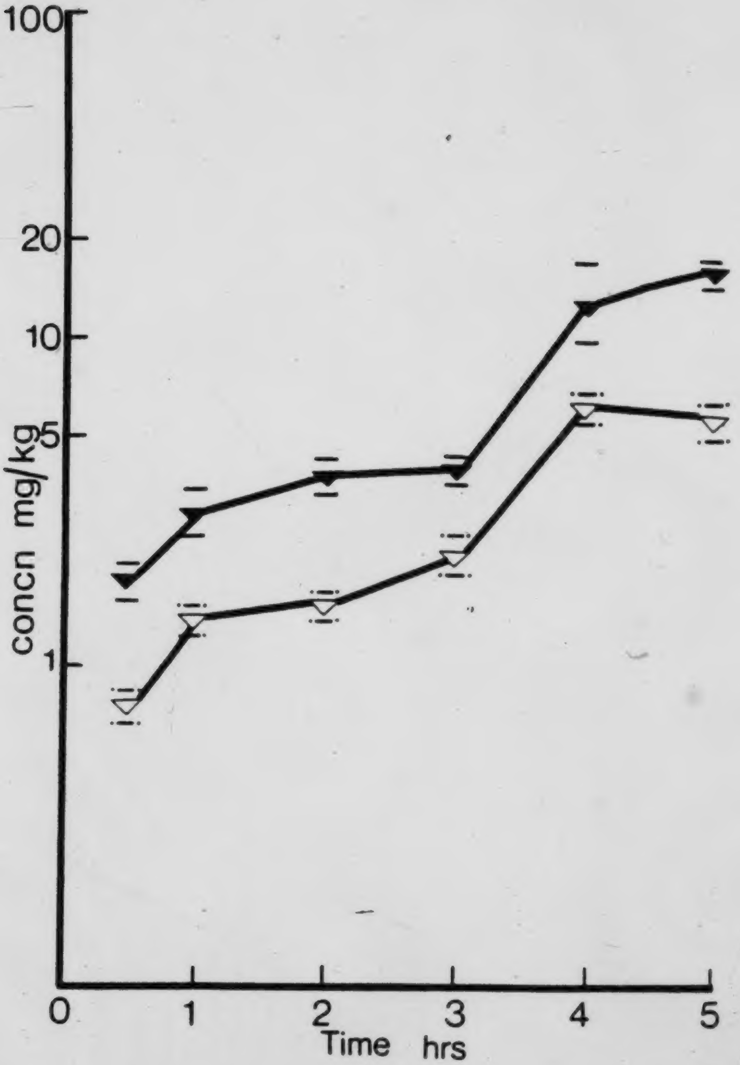


Figure 48. Rat liver levels of R(-) [▼] and S(+) [▽] norfenfluramine after intraperitoneal administration of racemic fenfluramine (40 mg/kg).

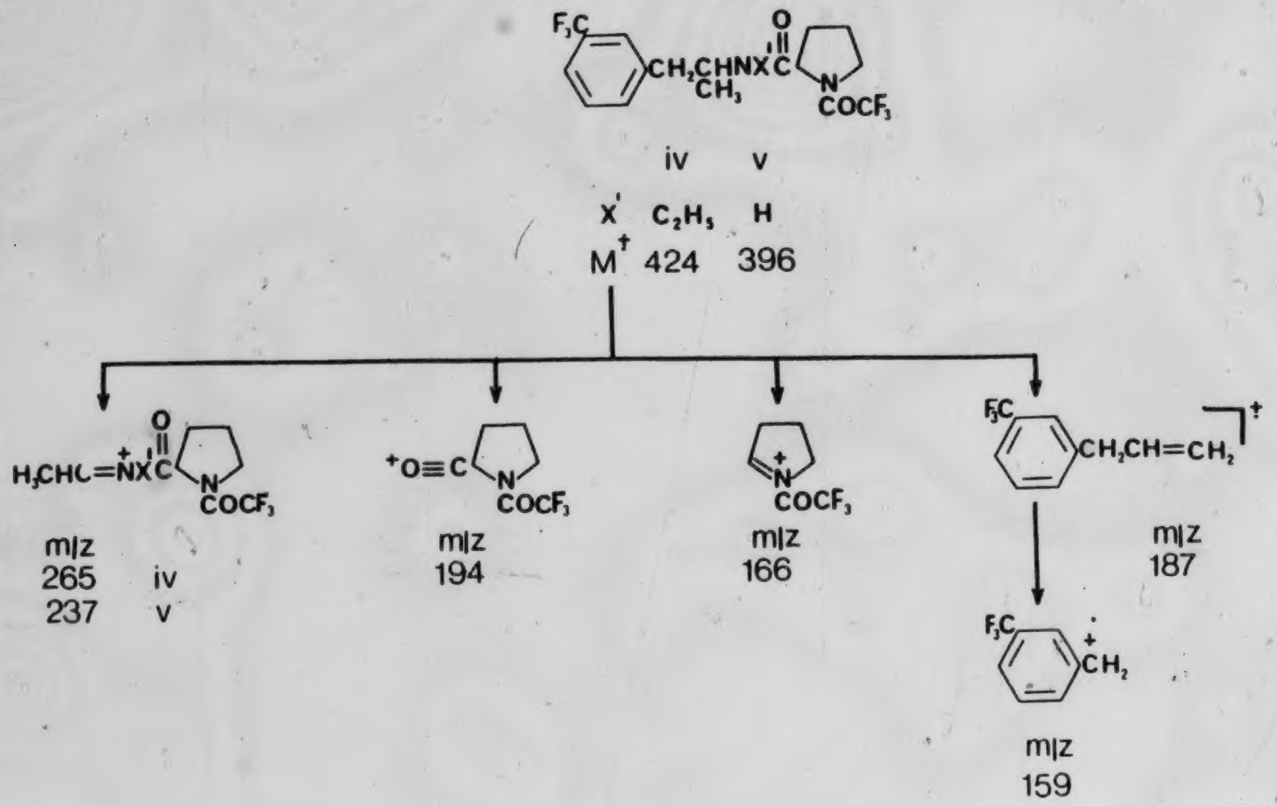

F3C
CH2CHNXC
CH3
O
N
COCF3
iv v
X' C2H5 H
M+ 424 396
H3CHC=NXC
m|z
265 iv
237 v
+O≡C
m|z
194
m|z
166
CH2CH=CH2
m|z
187
CH2
m|z
159

Figure 49. Proposed structures for fenfluramine (iv) and norfenfluramine (v) N-TPC MS fragments.

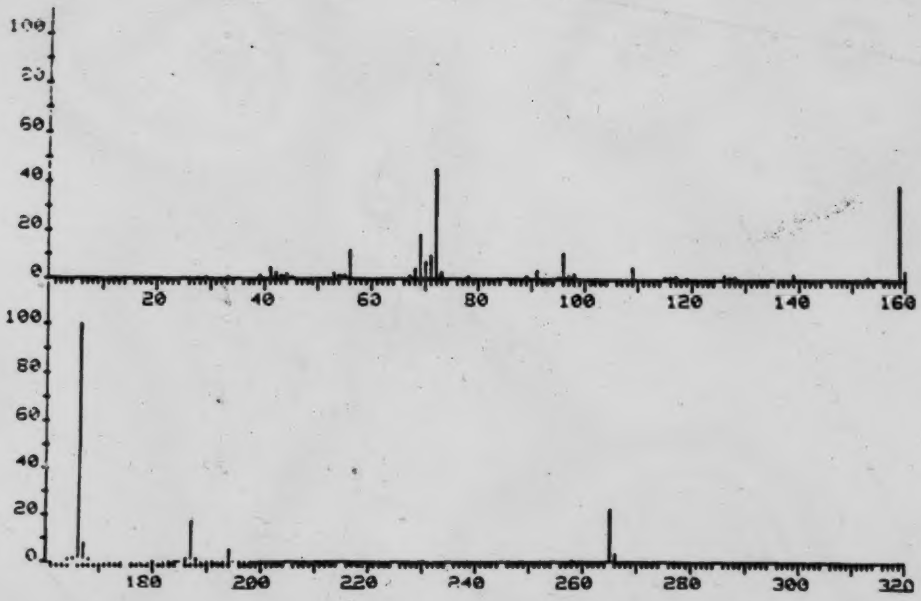


Figure 50. MS-line diagram of R,S-fenfluramine-N-TPC.

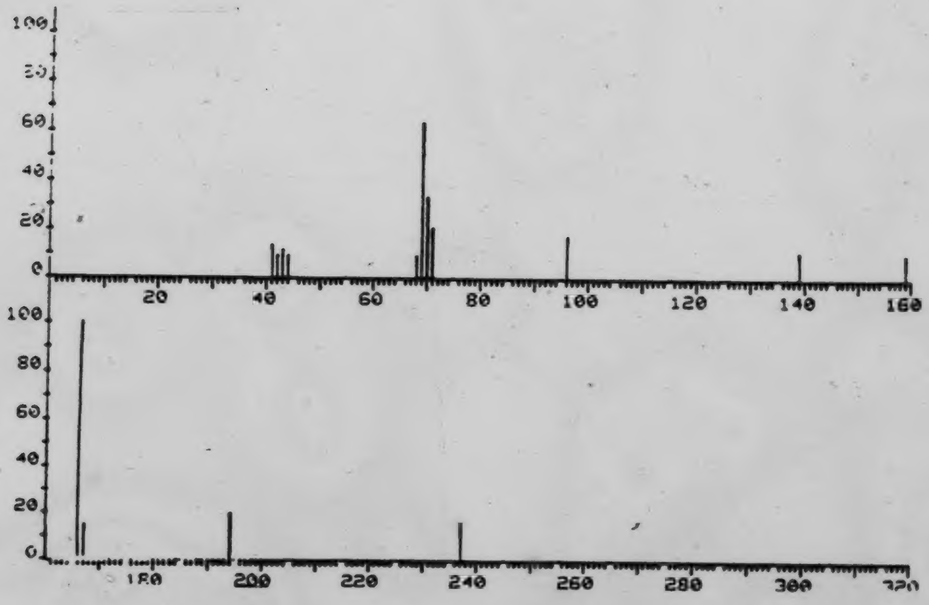


Figure 51. MS-line diagram of R,S-norfenfluramine-N-TPC.

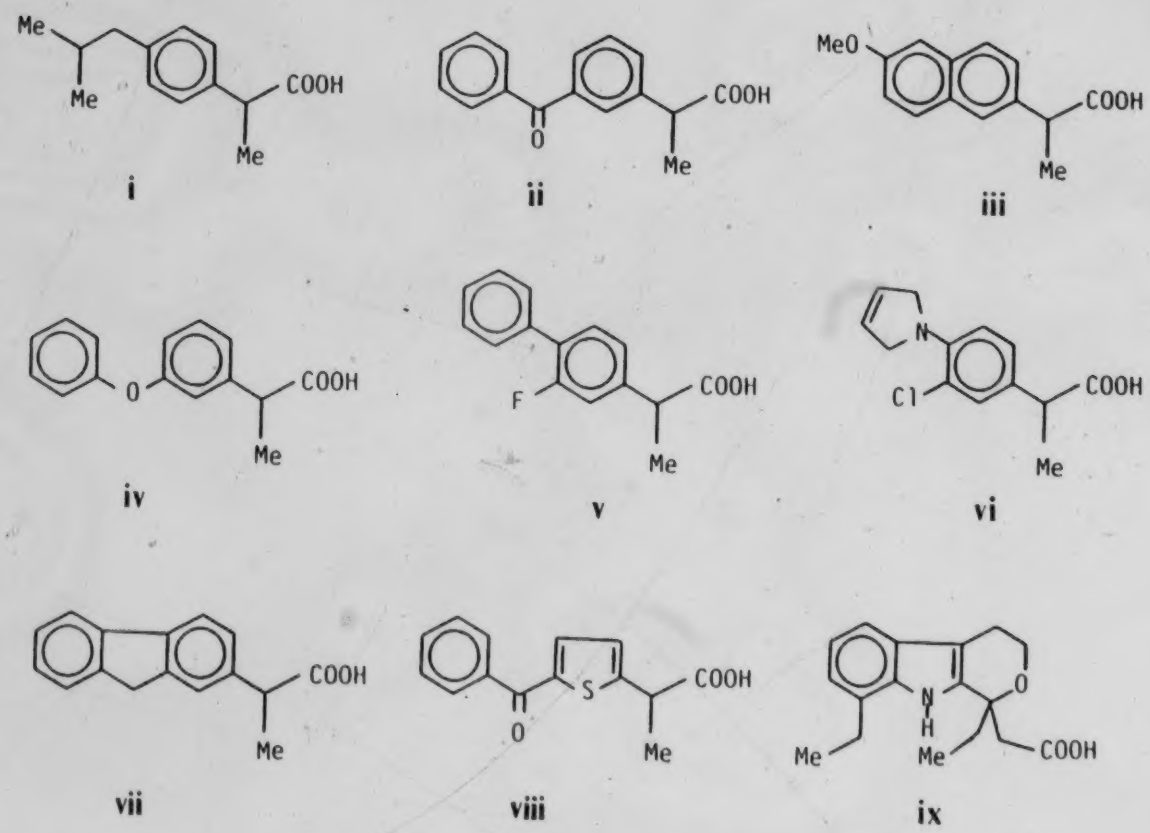

Me
Me
COOH
Me
i
O
COOH
Me
ii
MeO
COOH
Me
iii
O
COOH
Me
iv
F
COOH
Me
v
N
Cl
COOH
Me
vi
COOH
Me
vii
O
S
COOH
Me
viii
N
H
O
Me
Me
COOH
ix

Figure 52. Structures of arylalkanoic non-steroidal antiinflammatory drugs. Ibuprofen (i); ketoprofen (ii); naproxen (iii); fenoprofen (iv); flurbiprofen (v); pirprofen (vi); cicloprofen (vii); tiaprofenic acid (viii); etodolac (ix).

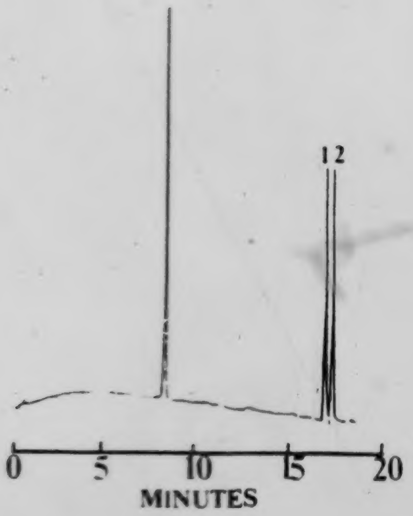


Figure 53. Gas chromatogram of (R,S)-ibuprofen, S(+)-α-methylbenzene-ethanamine derivatives prepared via acid chloride intermediate. Key: 1 = S(+)-ibuprofen; 2 = R(-)-ibuprofen.

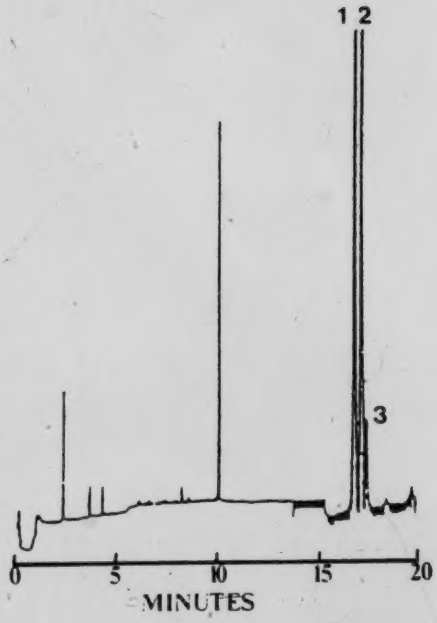


Figure 54. Gas chromatogram of (R,S)-ibuprofen, S(+)-α-methylbenzene-ethanamine derivatives prepared by condensation with 10 ug CDI. Key: 1 = S(+)-ibuprofen; 2 = R(-)-ibuprofen; 3 = unknown component.

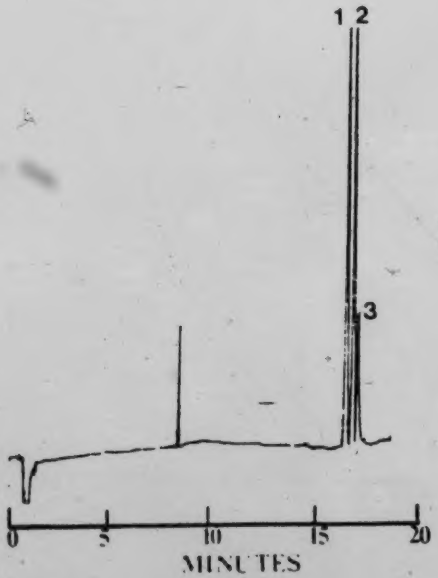


Figure 55. Gas chromatogram of (R,S)-ibuprofen, R(-)-α-methylbenzene-ethanamine derivatives prepared by condensation with 10 ug CDI. Key: 1 = R(-)-ibuprofen; 2 = S(+)-ibuprofen; 3 = unknown component.

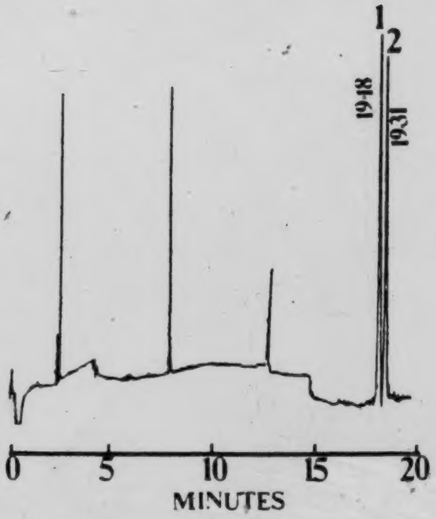


Figure 56. Gas chromatogram of (R,S)-fenoprofen, S(+)-α- methylbenzene-ethanamine derivatives prepared by condensation with 10 ug CDI.
Key: 1 = S(+)-fenoprofen; 2 = R(-)-fenoprofen.

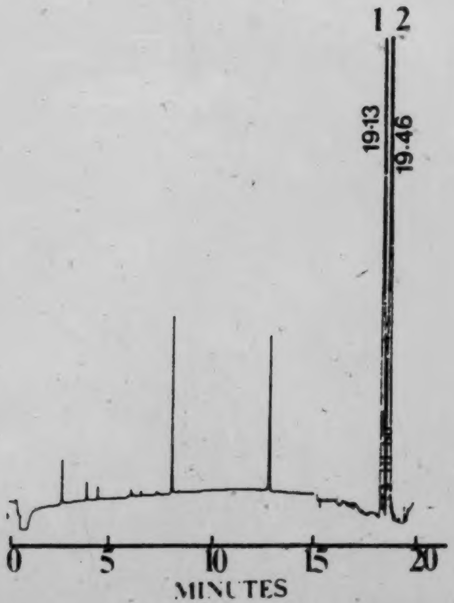


Figure 57. Gas chromatogram of (R,S)-fenoprofen, R(-)- α-methylbenzene-ethanamine derivatives prepared by condensation with 10 ug CDI.
Key: 1 = R(-)-fenoprofen; 2 = S(+)-fenoprofen.

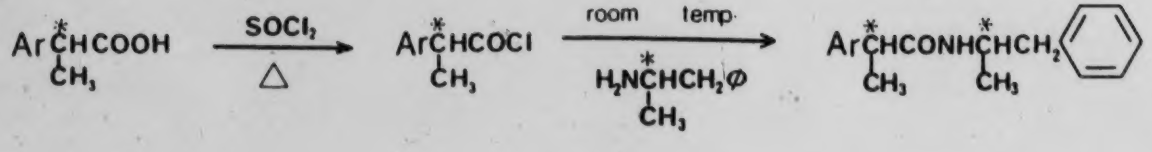

ArCHCOOH
CH3
SOCl2
△
ArCHCOCl
CH3
room temp
H2NCHCH2Φ
CH3
ArCHCONHCHCH2
CH3
CH3

Figure 58. Scheme for preparation of α-methylbenzeneethanamines via acid chloride acylation of arylalkanoic acids; * denotes an asymmetric center.

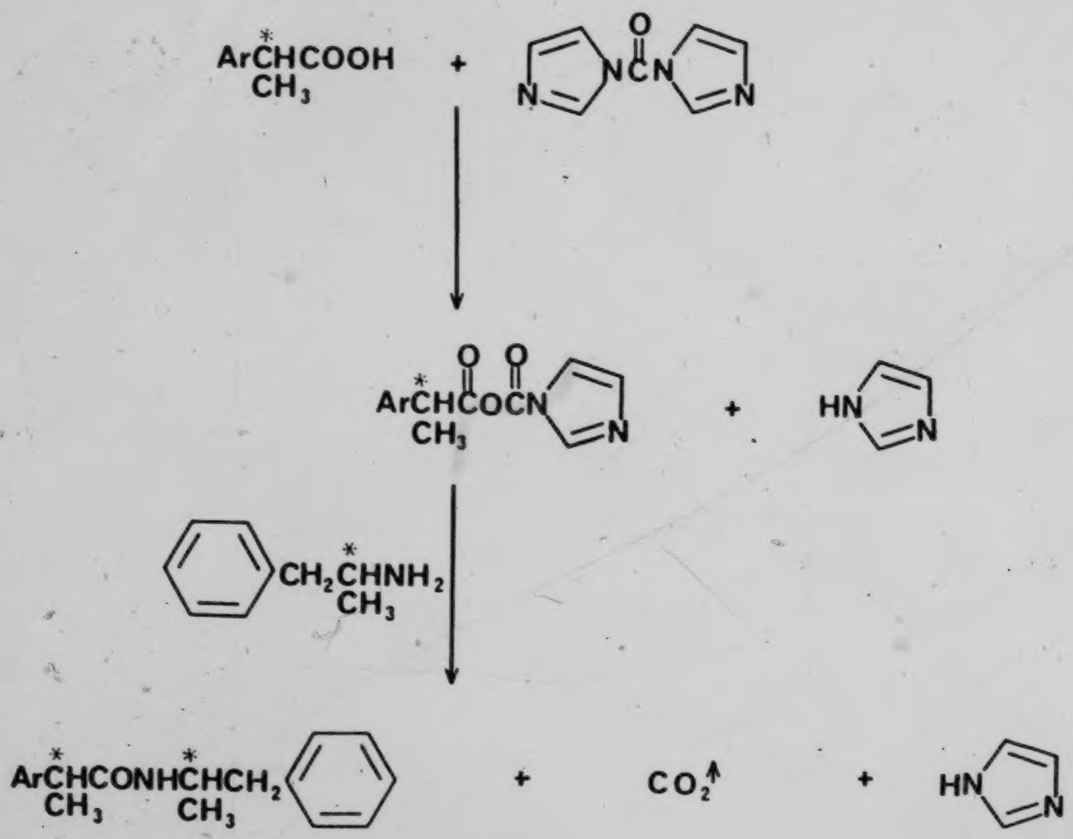

ArCHCOOH
CH3
+
O
NCN
N
N
O O
ArCHCOCN
CH3
N
+
HN
N
CH2CHNH2
CH3
ArCHCONHCHCH2
CH3 CH3
+
CO2
+
HN
N
*

Figure 59. Scheme for preparation of α -methylbenzeneethanamides by condensation of arylalkanoic acids with CDI; * denotes an asymmetric center.

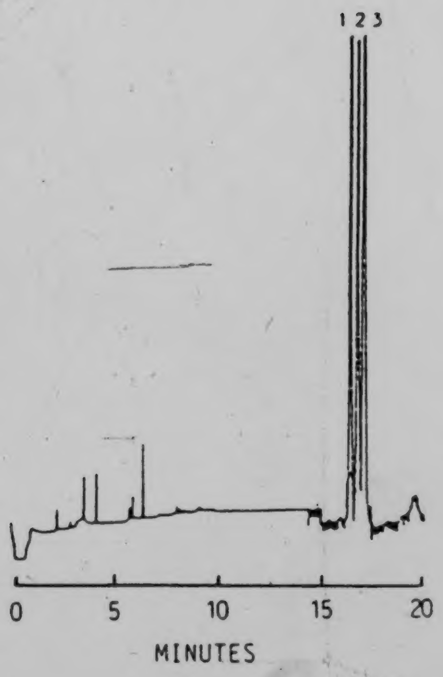


Figure 60. Gas chromatographic resolution of (R,S)-ibuprofen with S(+)-AMP after reaction with 50 ug CDI.
Key: 1 = S(+)-ibuprofen; 2 = R(-)-ibuprofen; 3 = unidentified component.

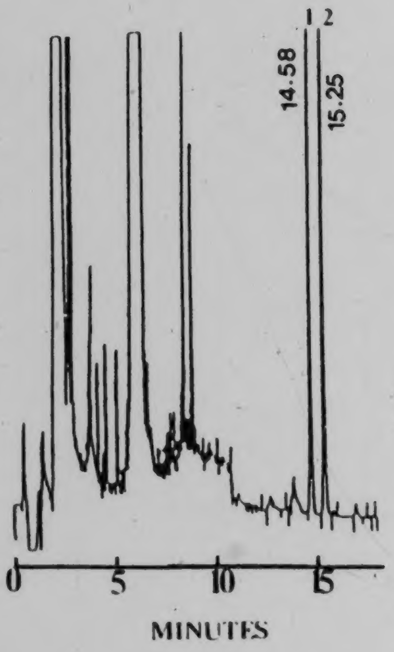


Figure 61. Gas chromatographic resolution of (R,S)-ibuprofen with S(-)-MBA.

Key: 1 = S(+)-ibuprofen; 2 = R(-)-ibuprofen.

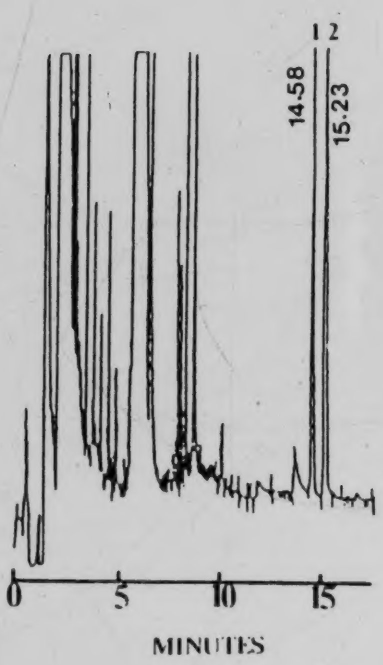


Figure 62. Gas chromatographic resolution of (R,S)-ibuprofen with R(+)-MBA.

Key: 1 = R(-)-ibuprofen; 2 = S(+)-ibuprofen.

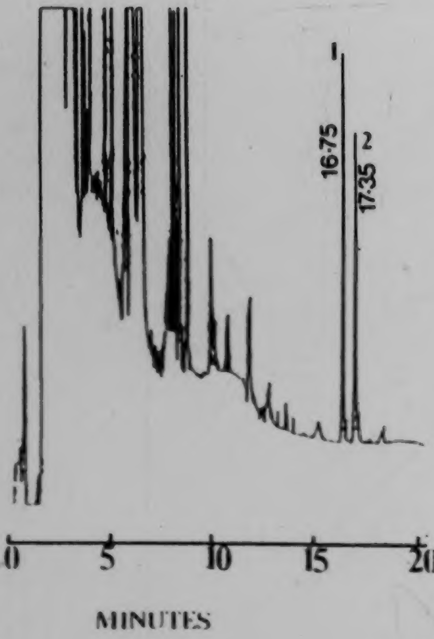


Figure 63. Gas chromatographic resolution of (R,S)-fenoprofen with S(-)-MBA. Key: 1 = S(+)-fenoprofen; 2 = R(-)-fenoprofen.

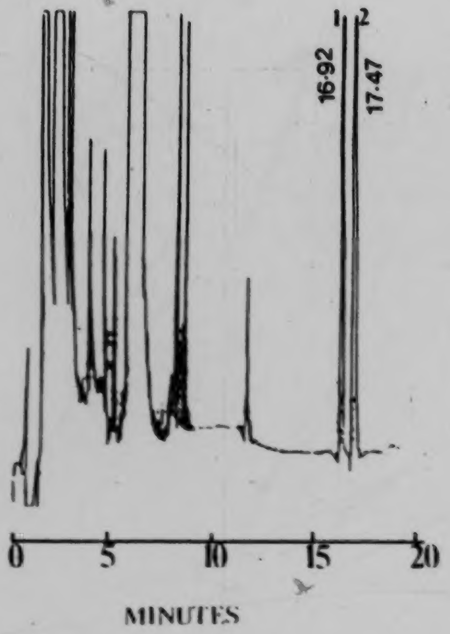


Figure 64. Gas chromatographic resolution of (R,S)-fenoprofen with R(+)-MBA. Key: 1 = R(-)-fenoprofen; 2 = S(+)-fenoprofen.

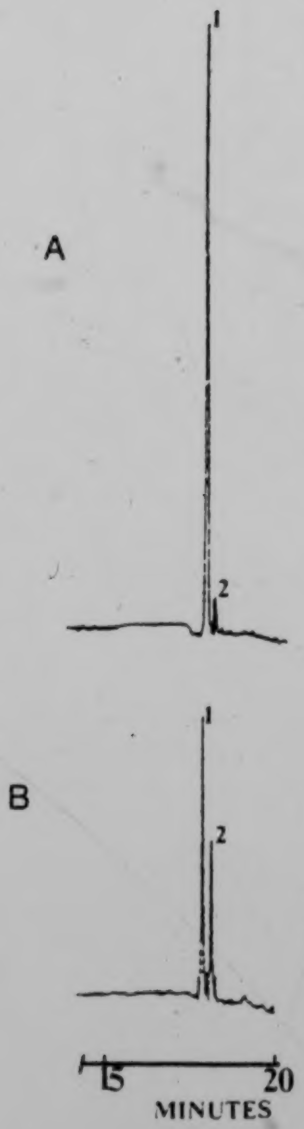


Figure 65. Gas chromatograms of S(+)-amphetamine derivatives of S(+)-ketoprofen (A) and racemic ketoprofen (B) enriched with the S(+)-isomer. Key: 1 = S(+)-ketoprofen; 2 = R(-)-ketoprofen.

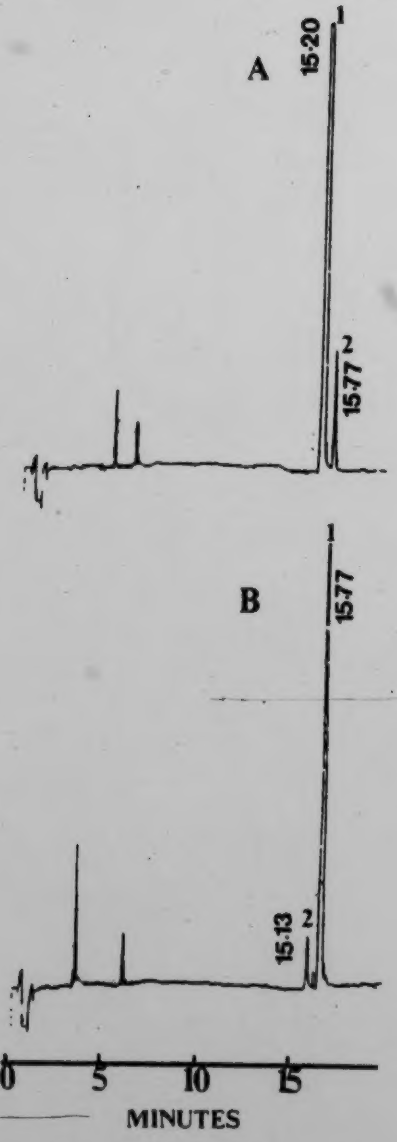


Figure 66. Gas chromatographic resolution of S(+)-naproxen, containing trace amounts of the R(-)-isomer, after reaction with S(+)-amphetamine (A) and R(-)-amphetamine (B). Key; 1 = S(+)-naproxen; 2 = R(-)-naproxen.

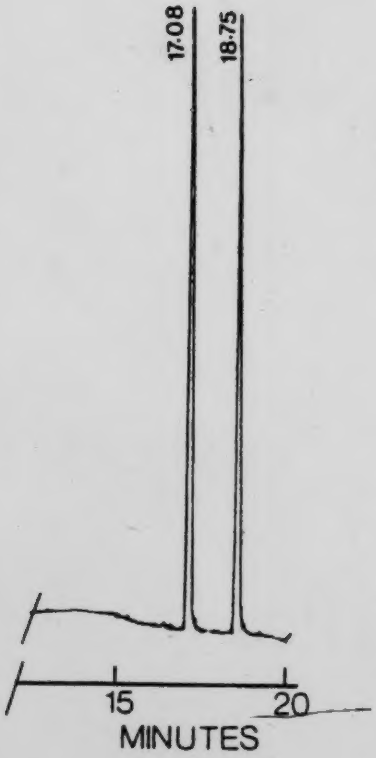


Figure 67. GC trace of (+/-)-ET, S(+)-AMP diastereoisomers. First peak (17.08 min) corresponds to (+)-ET and the second to (-)-ET.

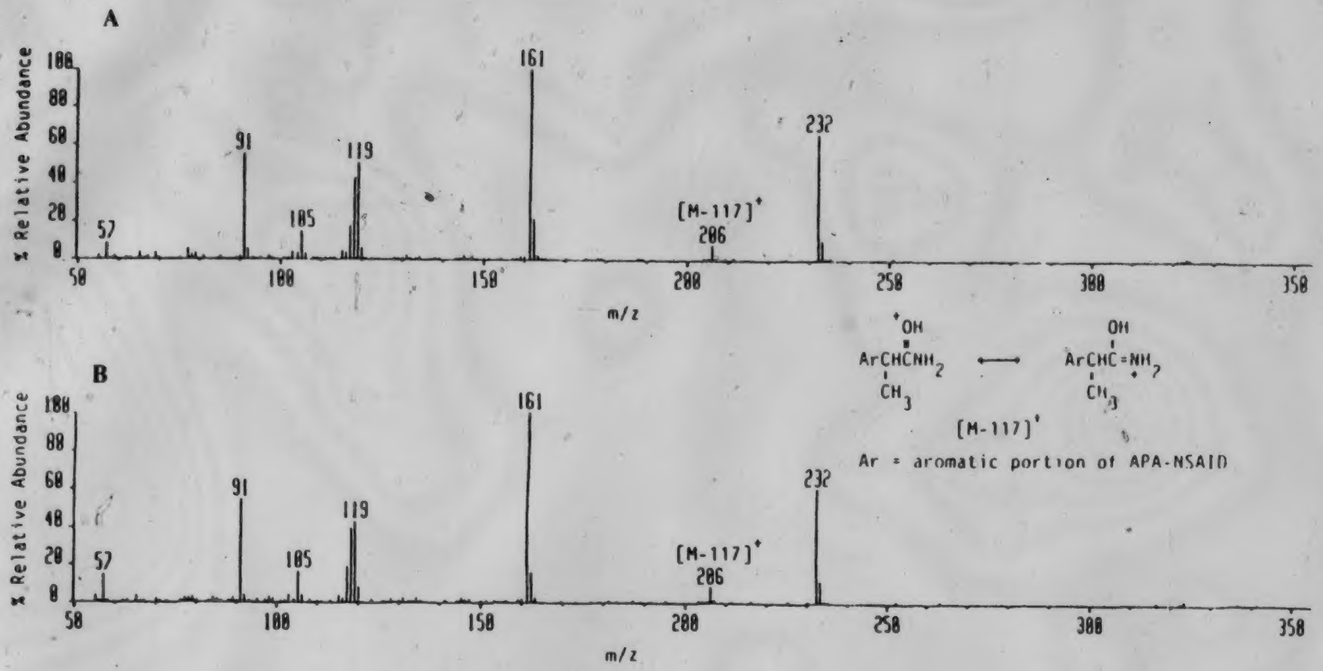


Figure 68. Mass spectra of ibuprofen, S(+)-AMP diastereomers. Key: S-ibuprofen, S-AMP (A); R-ibuprofen, S-AMP (B).

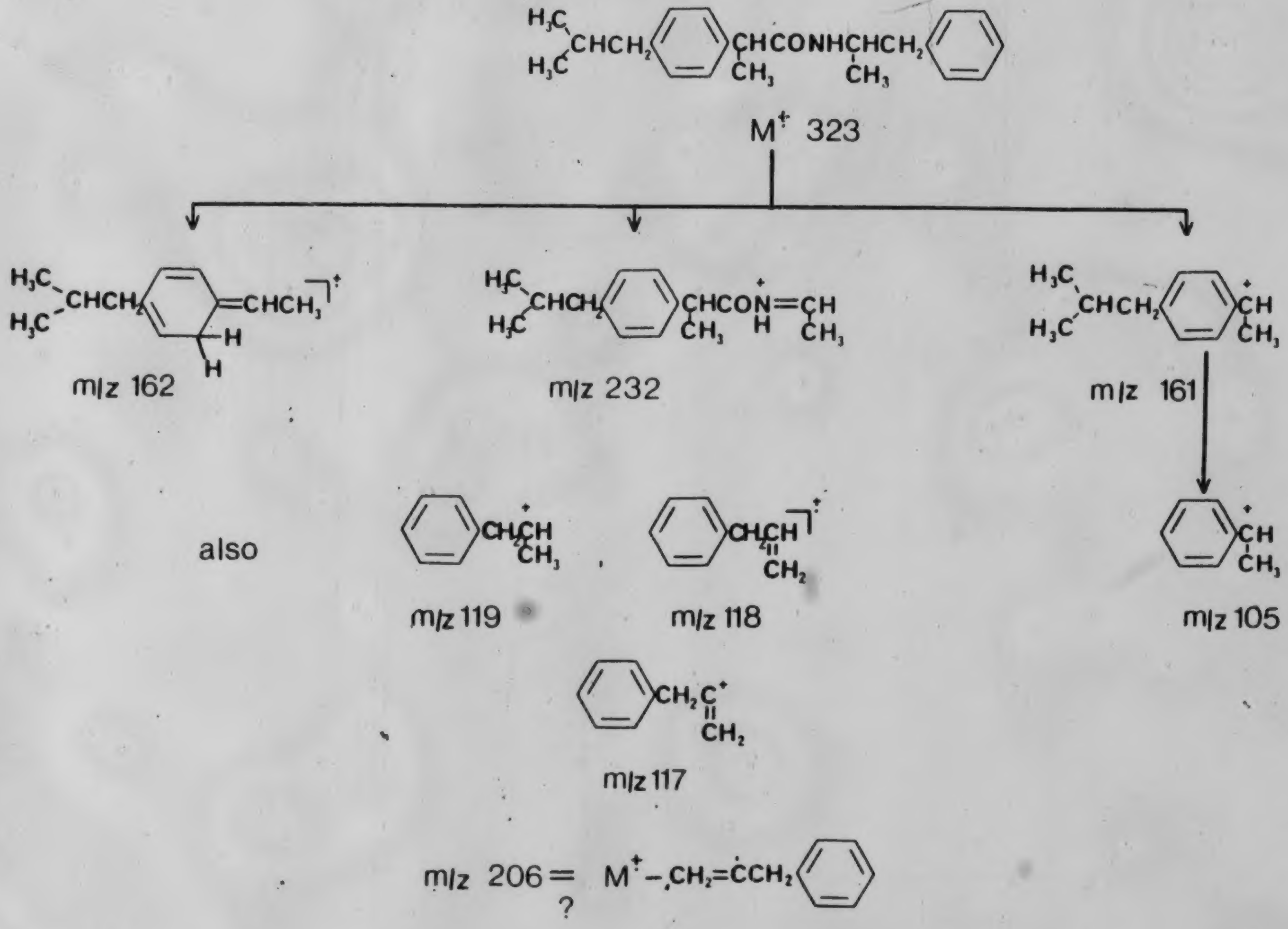

M⁺ 323
m/z 162
m/z 232
m/z 161
also
m/z 119
m/z 118
m/z 105
m/z 117
m/z 206 = M⁺ – ·CH₂=ĊCH₂
?

Figure 69. Postulated structures for the MS fragments of diastereomeric ibuprofen, benzeneethanamides.

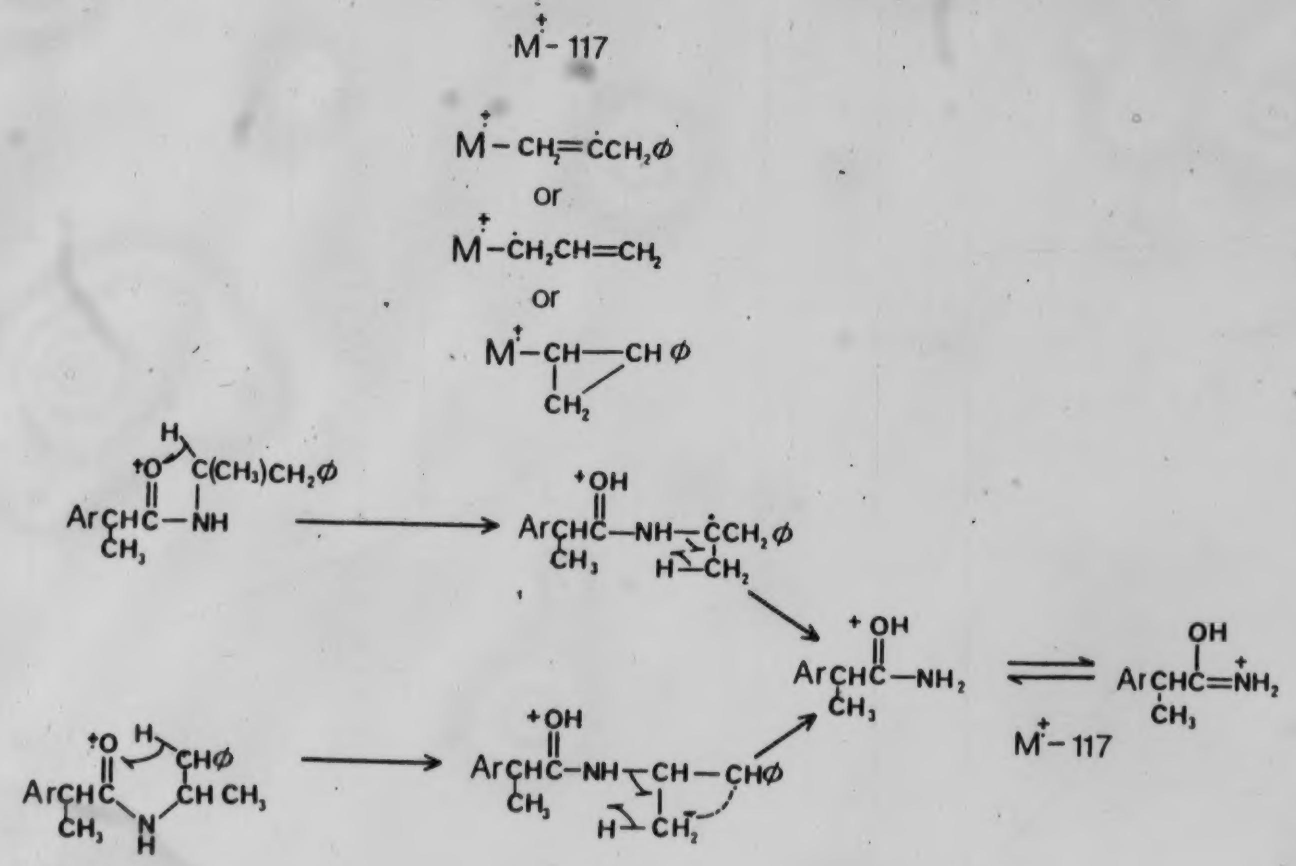

M⁺ - 117
M⁺ - CH2=ĊCH2Ø
or
M⁺ -ĊH2CH=CH2
or
M⁺ -CH—CHØ
CH2
ArCHC—NH
C(CH3)CH2Ø
ArCHC—NH—ĊCH2Ø
ArCHC—NH2
ArCHC=NH2
CHØ
CH CH3
ArCHC—NH—CH—CHØ
M⁺ - 117

Figure 70. Probable mechanisms for the formation of the [M-117] fragment observed in the mass spectra of all APA, amphetamine diastereomers.

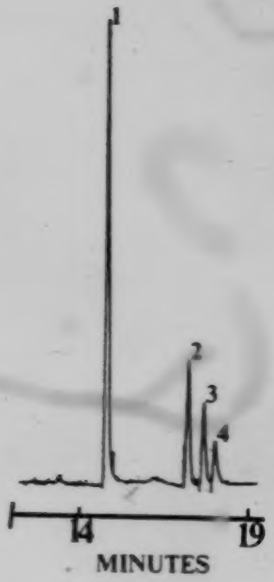


Figure 71. GC trace of S(+)-amphetamine-derivatized plasma extract obtained from a subject dosed (600 mg p.o.) with racemic ibuprofen (Motrin^R). Key: 1 = internal standard (p-methoxyphenylacetic acid); 2 = S(+)-ibuprofen; 3 = R(-)-ibuprofen; 4 = unknown component.

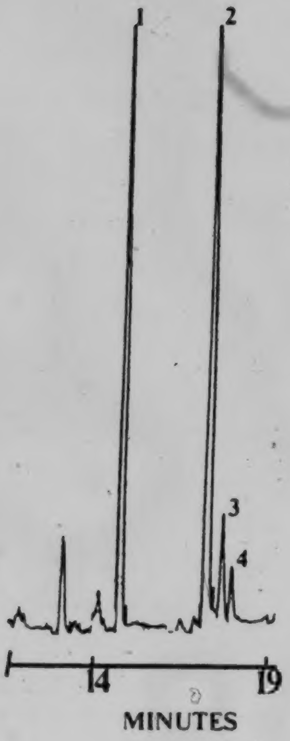


Figure 72. GC trace of S(+)-amphetamine-derivatized urine extract obtained from a subject dosed (600 mg p.o.) with racemic ibuprofen (Motrin^R). Key: 1 = internal standard (p-methoxyphenylacetic acid); 2 = S(+)-ibuprofen; 3 = R(-)-ibuprofen; 4 = unknown component.

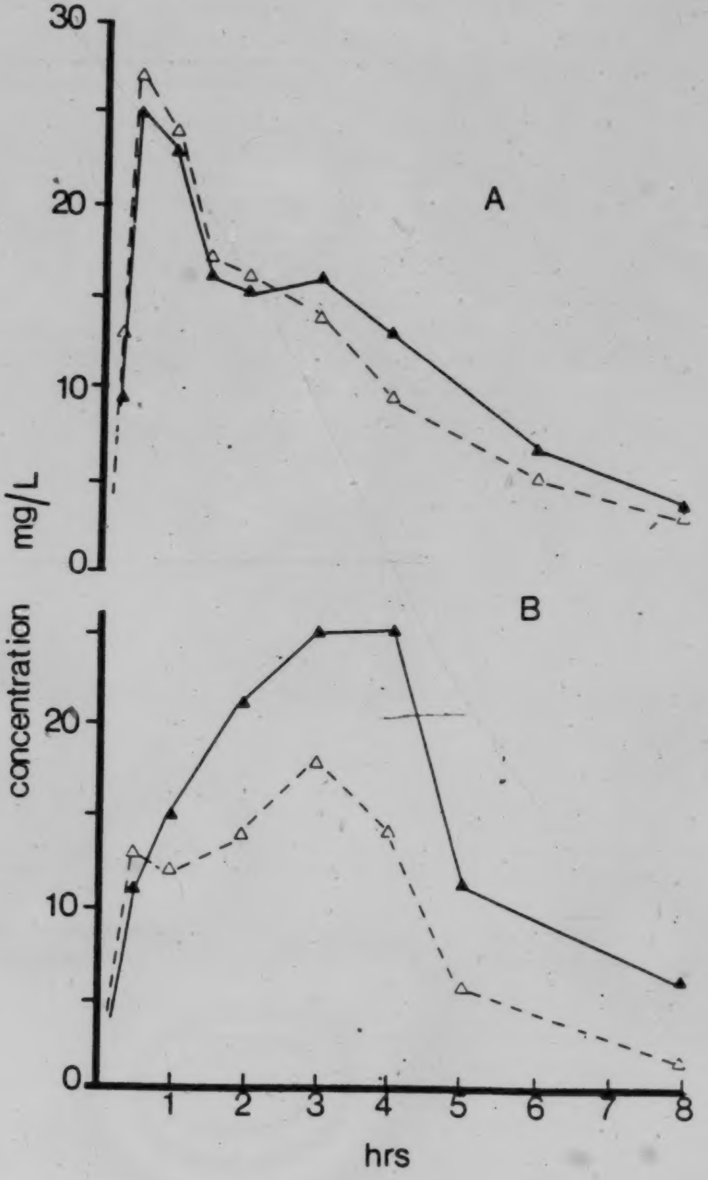


Figure 73. (R)-and (S)-ibuprofen plasma concentration-time curves for a subject dosed with Motrin^{R} (600 mg p.o.) (A) and Apo-Ibuprofen^{R}(B). Key: ▲ = S-isomer; △ = R-isomer.

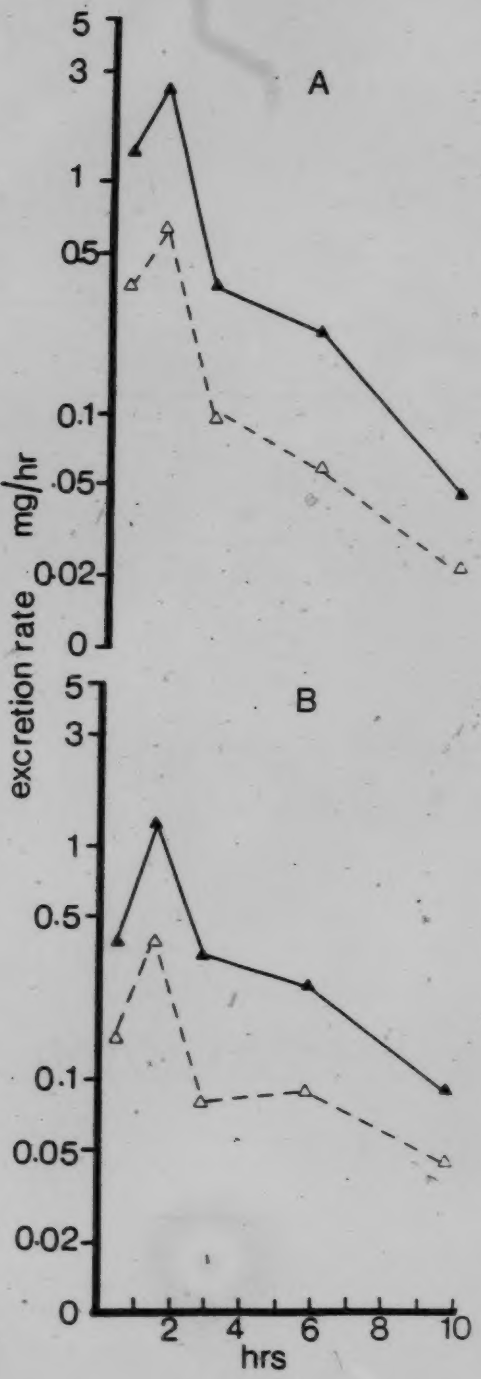


Figure 74. Urinary excretion rate curves of S-(▲) and R-(△) ibuprofen after administration (600 mg p.o.) of Motrin^R (A) and Apo-Ibuprofen^R (B).

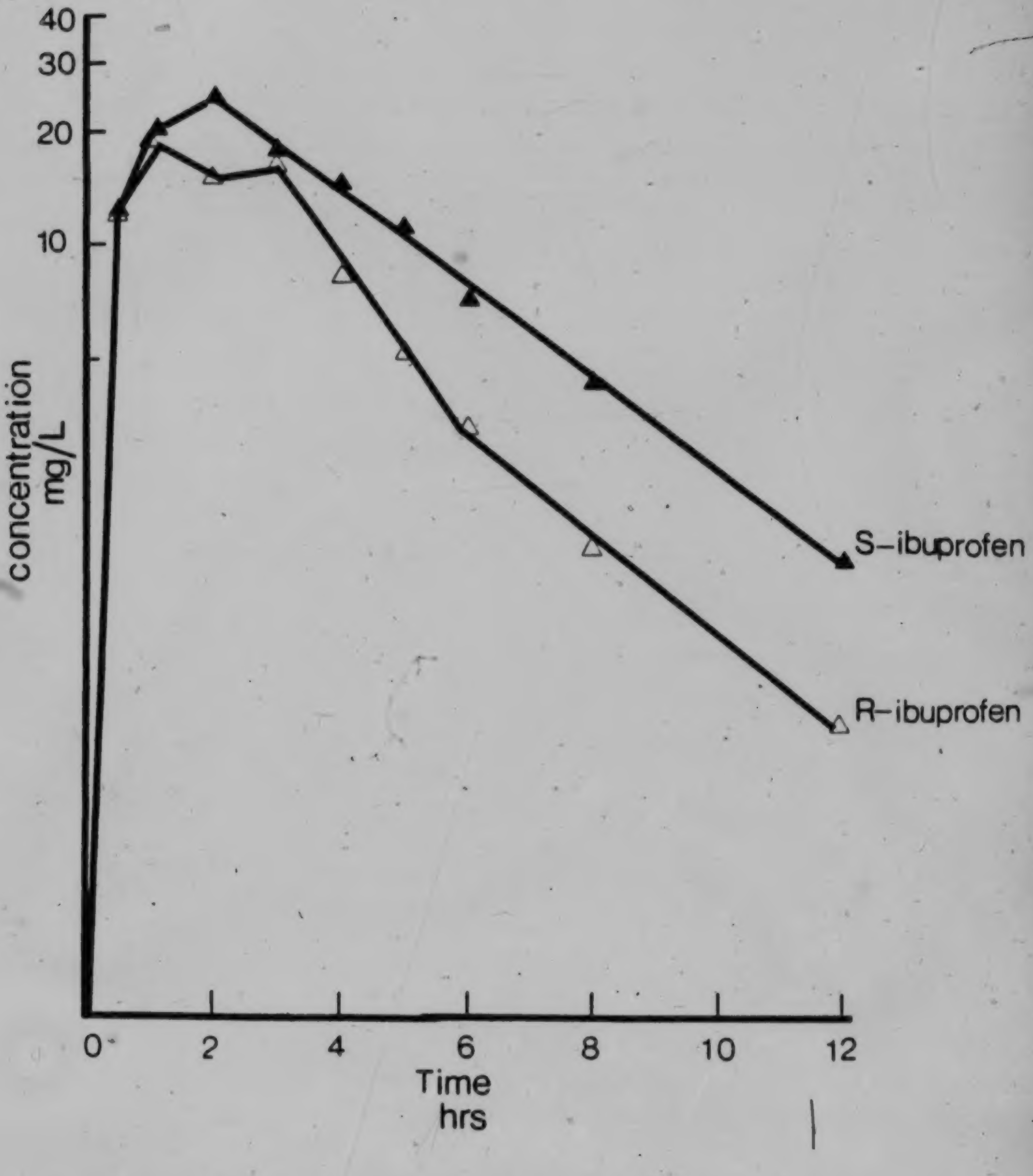


Figure 75. Mean plasma R- and S-ibuprofen concentration-time curves. Each point is a mean of 9 values.

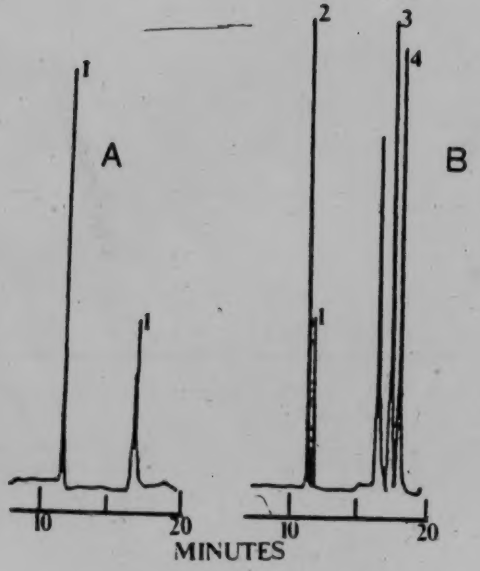


Figure 76. Gas chromatograms of plasma extracts from a subject dosed (200 mg p.o.) with tiaprofenic acid (Surgam^R). The derivatizing reagent was S(+)-amphetamine. (A) is the blank extract. Key (B): 1 = endogenous components; 2 = S(+)-naproxen (internal standard); 3 = R(+)-TA; 4 = S(-)-TA.

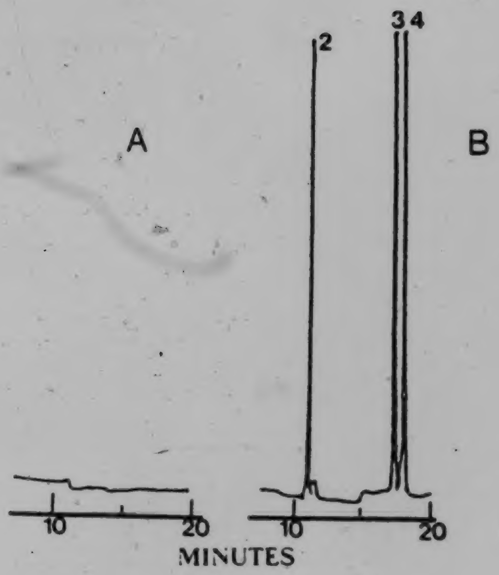


Figure 77. Gas chromatogram of urine samples from a subject dosed (200 mg p.o.) with tiaprofenic acid (Surgam^R). (A) is the blank extract. The derivatizing reagent was S(+)-amphetamine. Key (B): 2 = S(+)-naproxen; 3 = R(+)-TA; 4 = S(-)-TA.

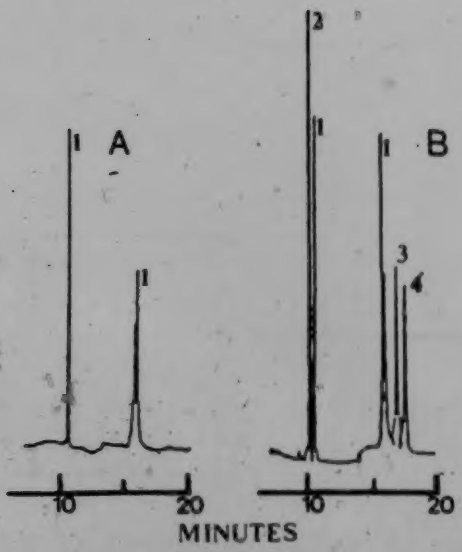


Figure 78. Gas chromatograms of synovial fluid extracts from a subject dosed (200 mg p.o.) with tiaprofenic acid (Surgam ^{R}). The derivatizing reagent was S(+)-amphetamine. (A) is the blank extract. Key (B): 1 = endogenous component; 2 = S(+)-naproxen; 3 = R(+)-TA; 4 = S(-)-TA.

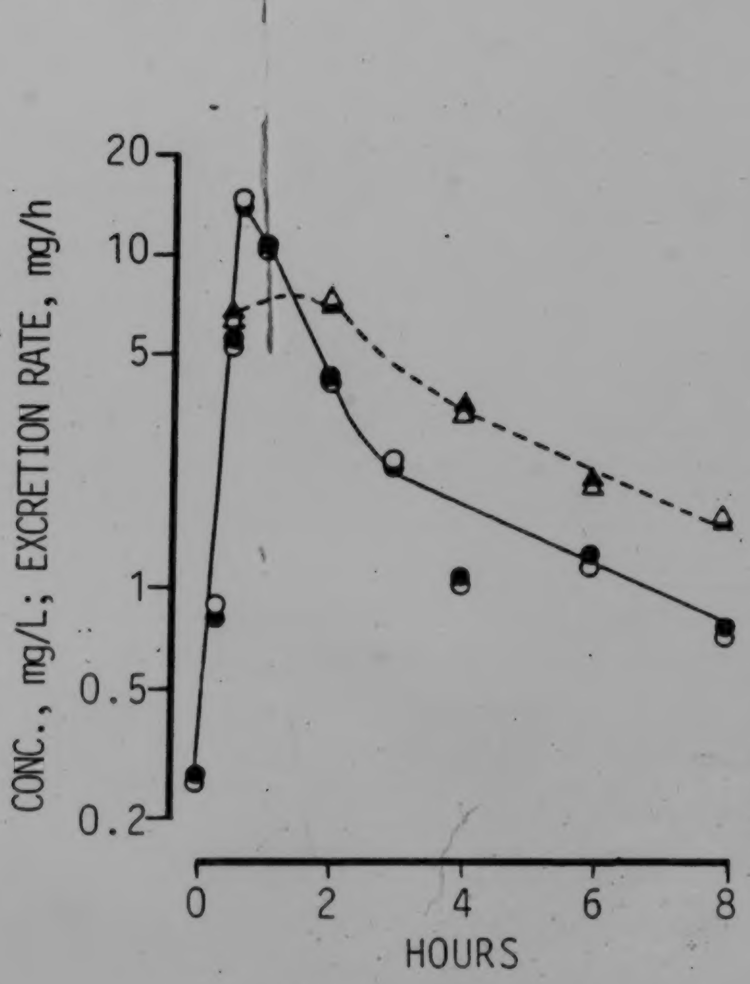


Figure 79. Steady-state plasma concentrations of tiaprofenic acid enantiomers (●,○) and urinary excretion rates of the enantiomer conjugates (▲,△) after 200 mg/8h doses to subject #4.
Key: (● ,▲) = R-isomer; (○,△) = S-isomer.

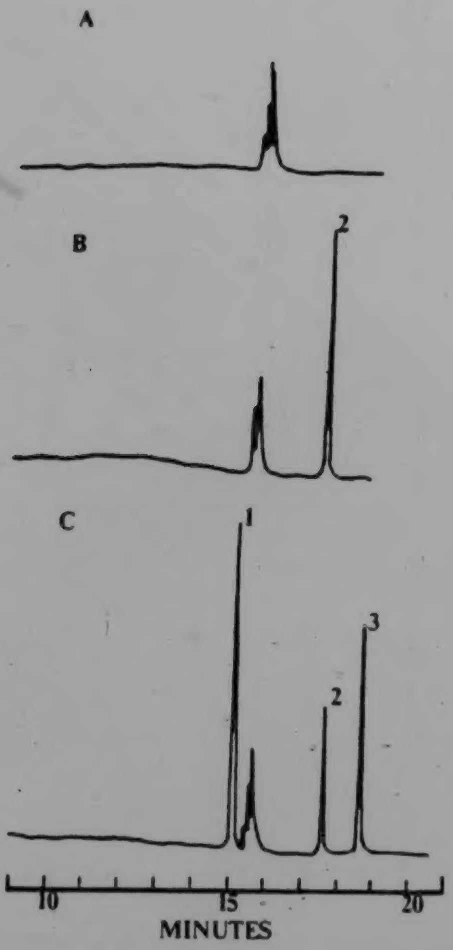


Figure 80. Gas chromatograms of S(+)-amphetamine-derivatized samples of blank plasma (A), plasma spiked with (+)-etodolac (B) and a plasma sample from a subject 1 hour after administration of racemic etodolac (C).
Key: 1 = S(+)-naproxen; 2 = (+)-etodolac; 3 = (-)-etodolac.

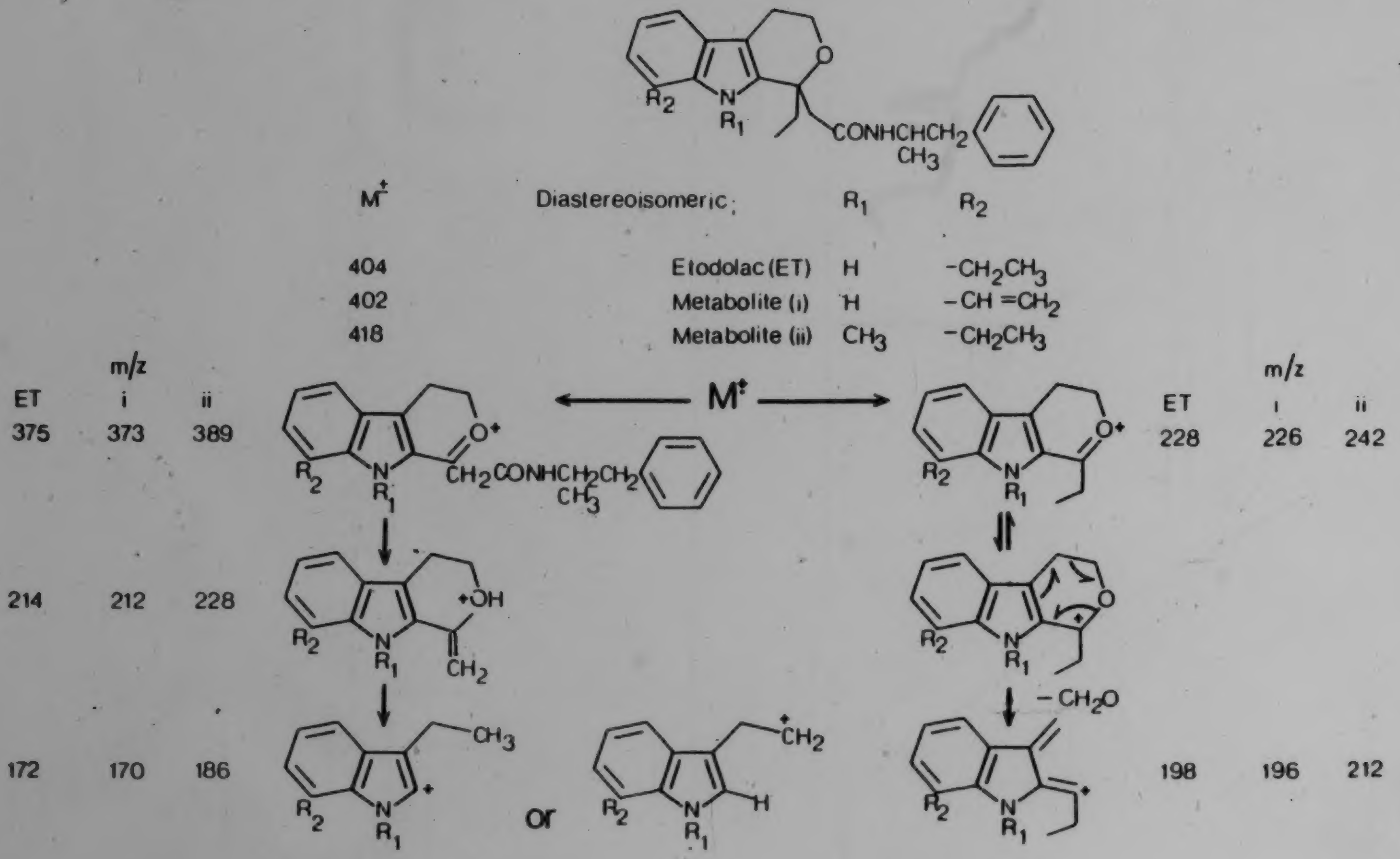

Diastereoisomeric;
R1
R2
M+
404
402
418
Etodolac (ET)
H
-CH2CH3
Metabolite (i)
H
-CH =CH2
Metabolite (ii)
CH3
-CH2CH3
m/z
ET
i
ii
375
373
389
214
212
228
172
170
186
M+
or
m/z
ET
i
ii
228
226
242
-CH2O
198
196
212

Figure 81. Postulated structures of MS fragments of etodolac (ET), metabolites (i) and (ii) after derivatization with S(+)-amphetamine.

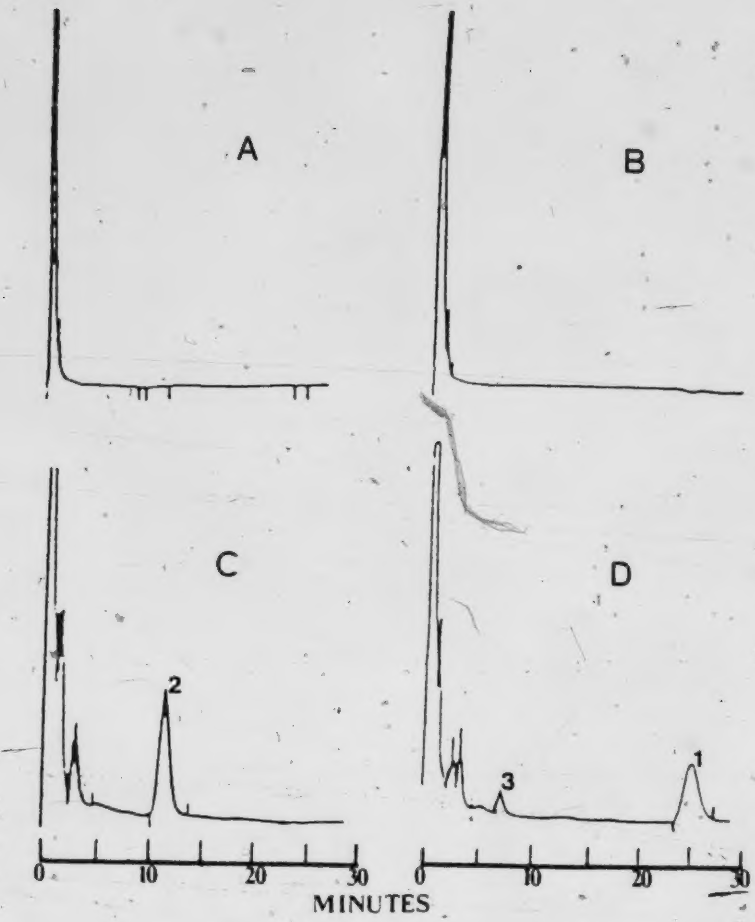


Figure 82. Liquid chromatogram of blank urine (A and B) and urine from a subject after a 200 mg p.o. dose of racemic etodolac before (A and C) and after (B and D) alkaline hydrolysis.
Key: 1 = etodolac; 2 = conjugated etodolac; 3 = dehydrogenated ET metabolite.

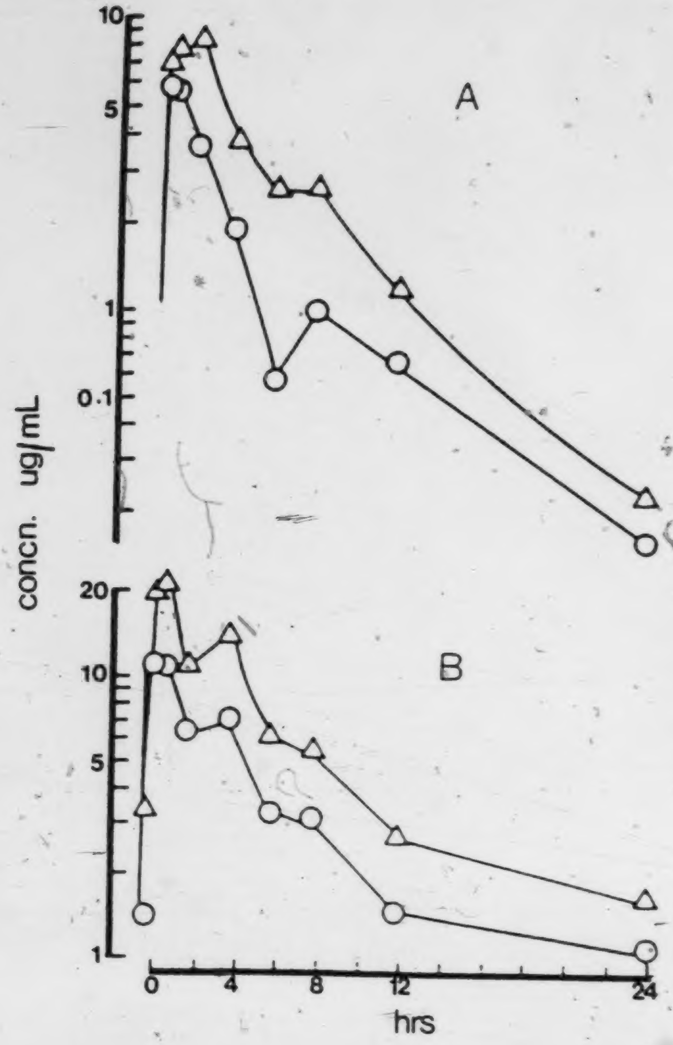


Figure 83. Plasma (+)-etodolac (○) and (-)-etodolac (△) concentration-time curves following administration of single (A) and repeated (B) 200 mg doses of racemic etodolac.

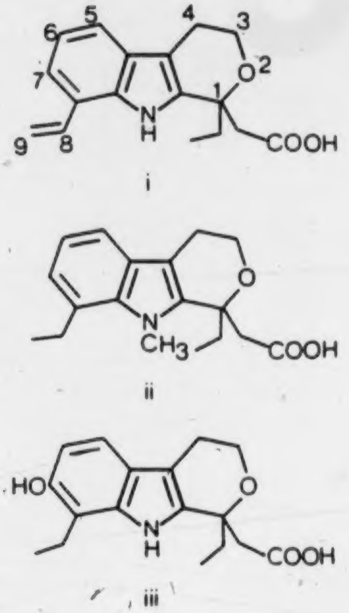

5
4
3
6
O2
7
1
N
H
9
8
COOH
i
O
N
CH3
COOH
ii
HO
O
N
H
COOH
iii

Figure 84. Structures of 8,9-dehydrogenated etodolac (i), N-methyletodolac (ii), and 7-hydroxyetodolac (iii).

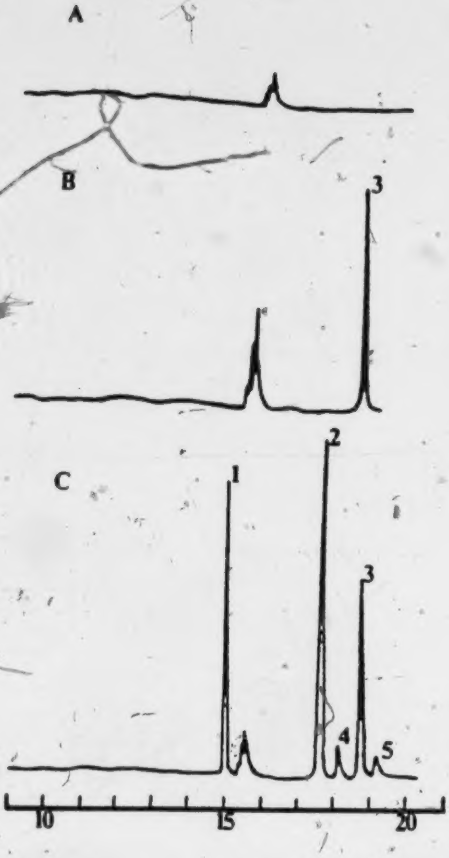


Figure 85. Gas chromatogram of blank urine (A), urine spiked with (-)-etodolac (B), and urine samples from a subject 1 hour after p.o. administration of 200 mg racemic etodolac. Key: 1 = S(+)-naproxen; 2 = (+)-etodolac; 3 = (-)-etodolac; 4 and 5 = 8,9-dehydrogenated metabolite isomers. S(+)-amphetamine was used as the derivatizing agent.

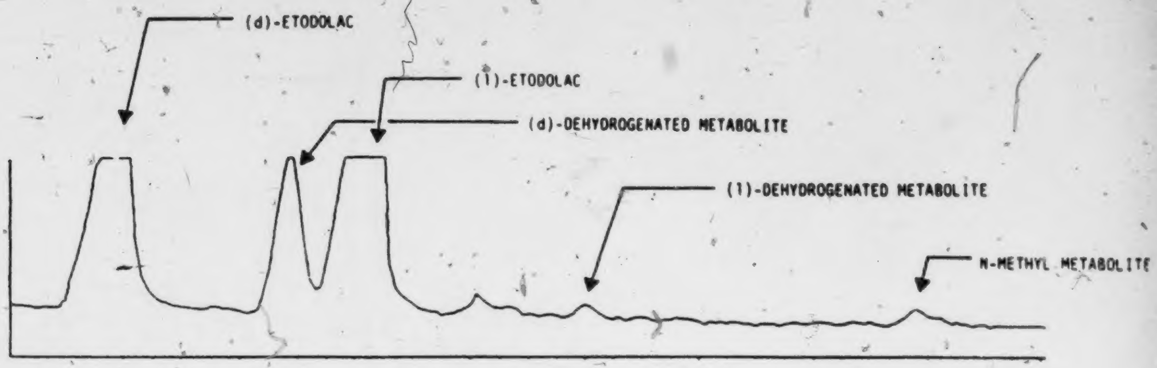


Figure 86. MS total ion trace containing etodolac, the dehydrogenated metabolite and the N-methyl metabolite.

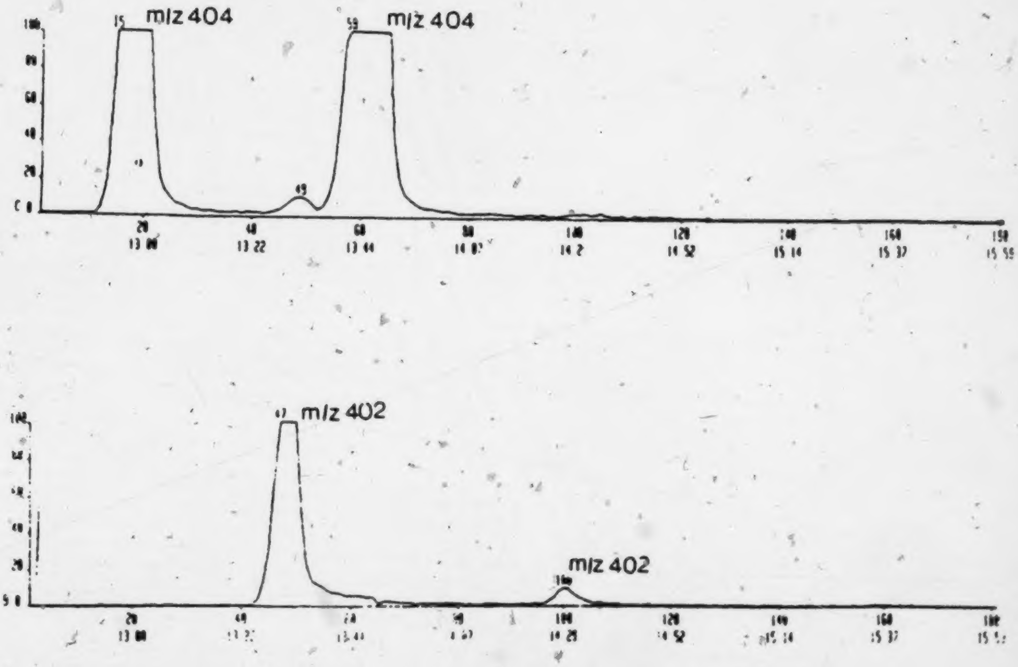


Figure 87. MS selected ion traces for the determination of the dehydrogenated metabolite of etodolac. m/z($m^{\cdot+}$) 404 characteristic for etodolac and m/z($m^{\cdot+}$) 402 characteristic for the dehydrogenated metabolite.

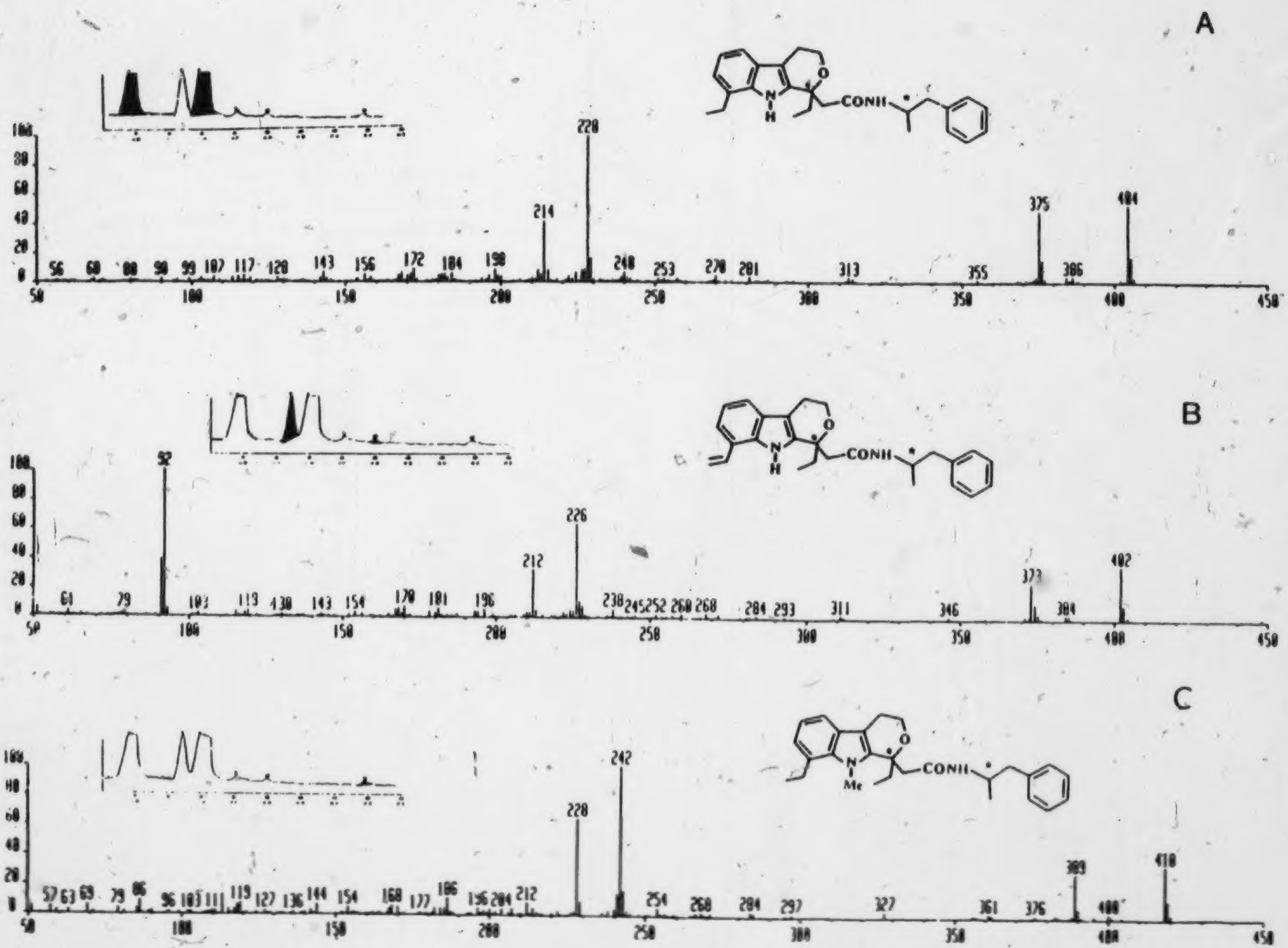


Figure 88. MS line diagrams for diastereomeric etodolac (A), 8,9-dehydrogenated etodolac (B) and N-methyletodolac (C).

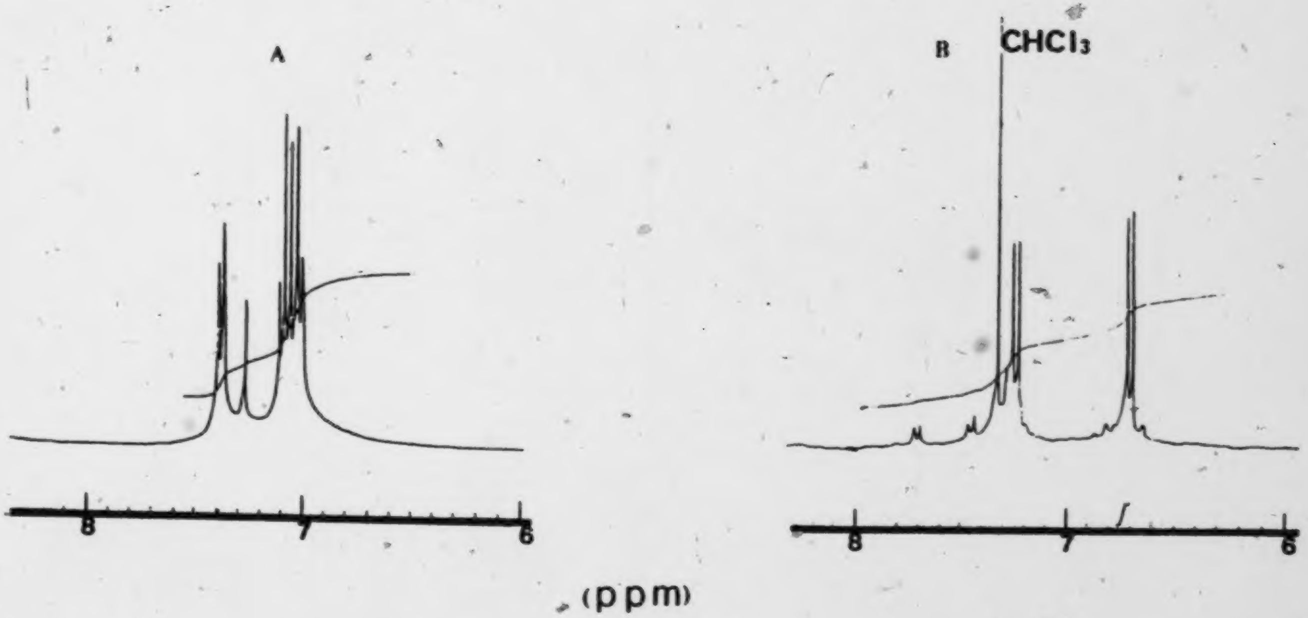


Figure 89. Partial proton NMR spectra of etodolac (A) and 7-hydroxyetodolac (B).

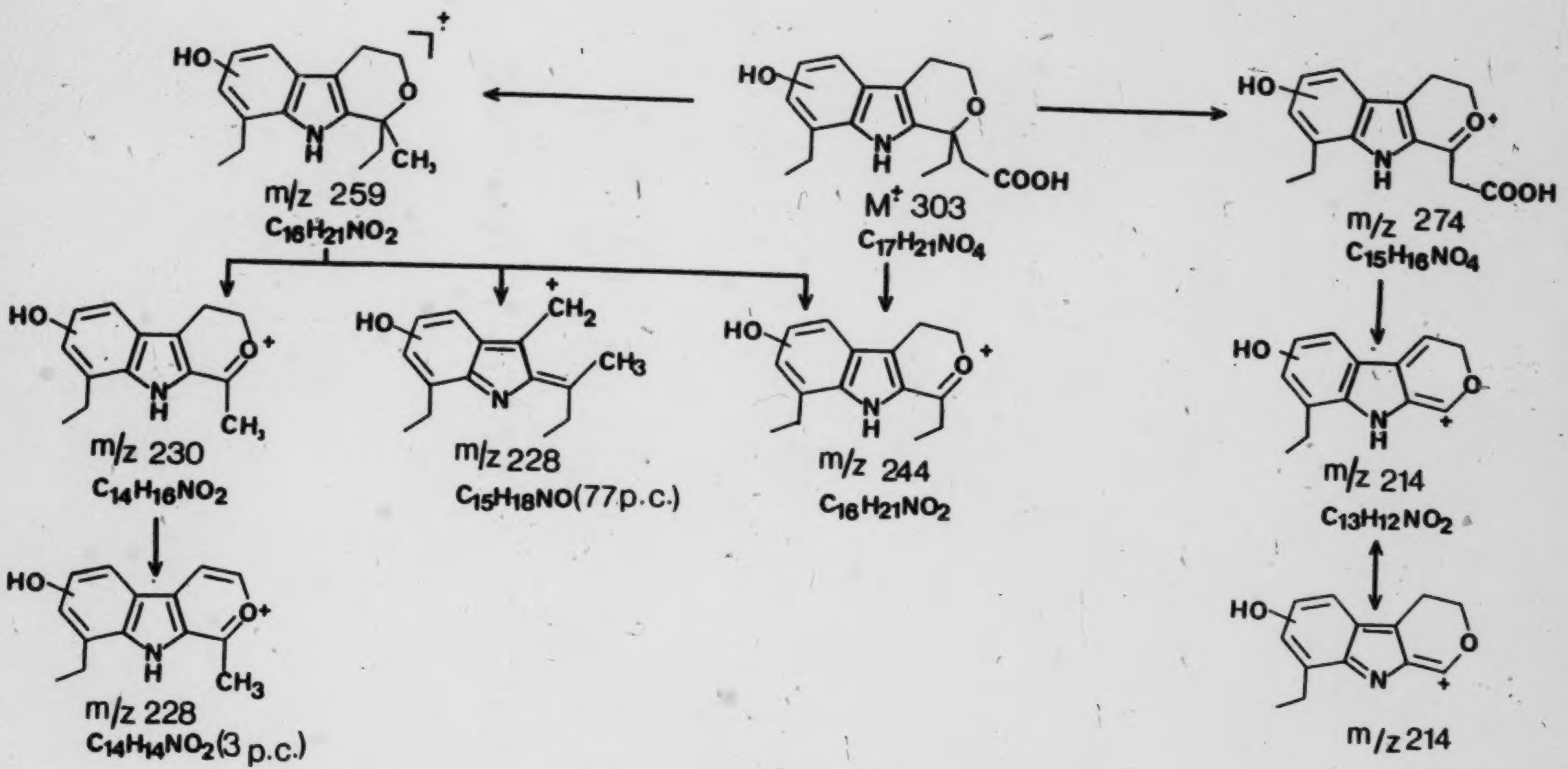

HO
M⁺ 303
C17H21NO4
COOH
m/z 259
C16H21NO2
CH3
m/z 274
C15H16NO4
m/z 230
C14H16NO2
m/z 228
C15H18NO(77 p.c.)
CH2
CH3
m/z 244
C16H21NO2
m/z 228
C14H14NO2(3 p.c.)
m/z 214
C13H12NO2
m/z 214
N
H
O

Figure 90. Postulated structures for DIP-MS fragments of 7-hydroxyetodolac isolated from urine and purified by column chromatography (silica gel, chloroform:ethanol 9.8:1.2).

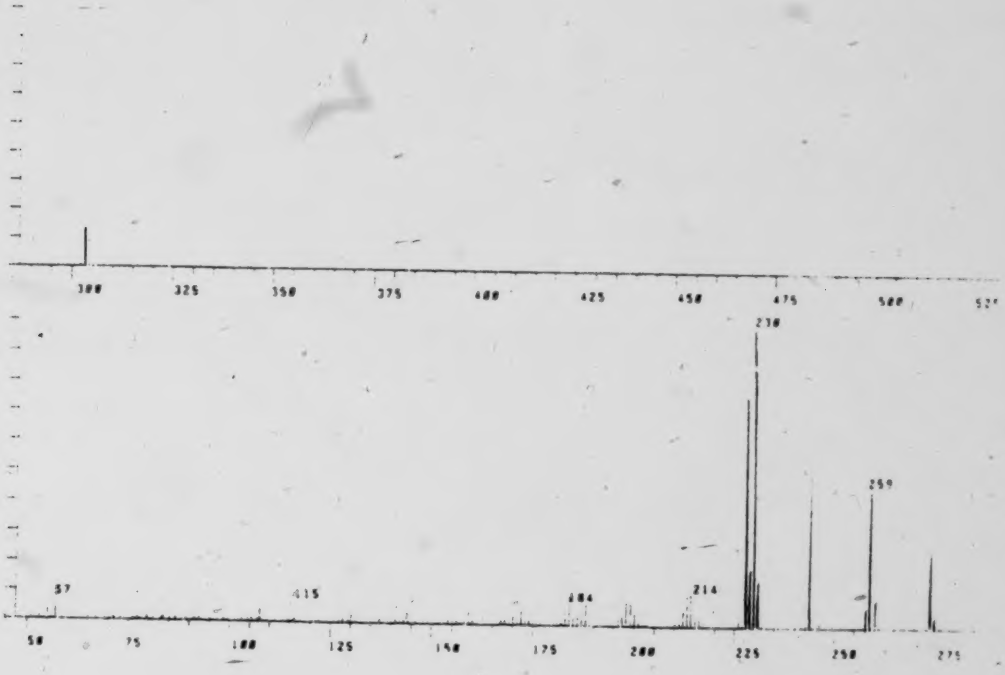


Figure 91. DIP-MS line diagram of 7-hydroxyetodolac.

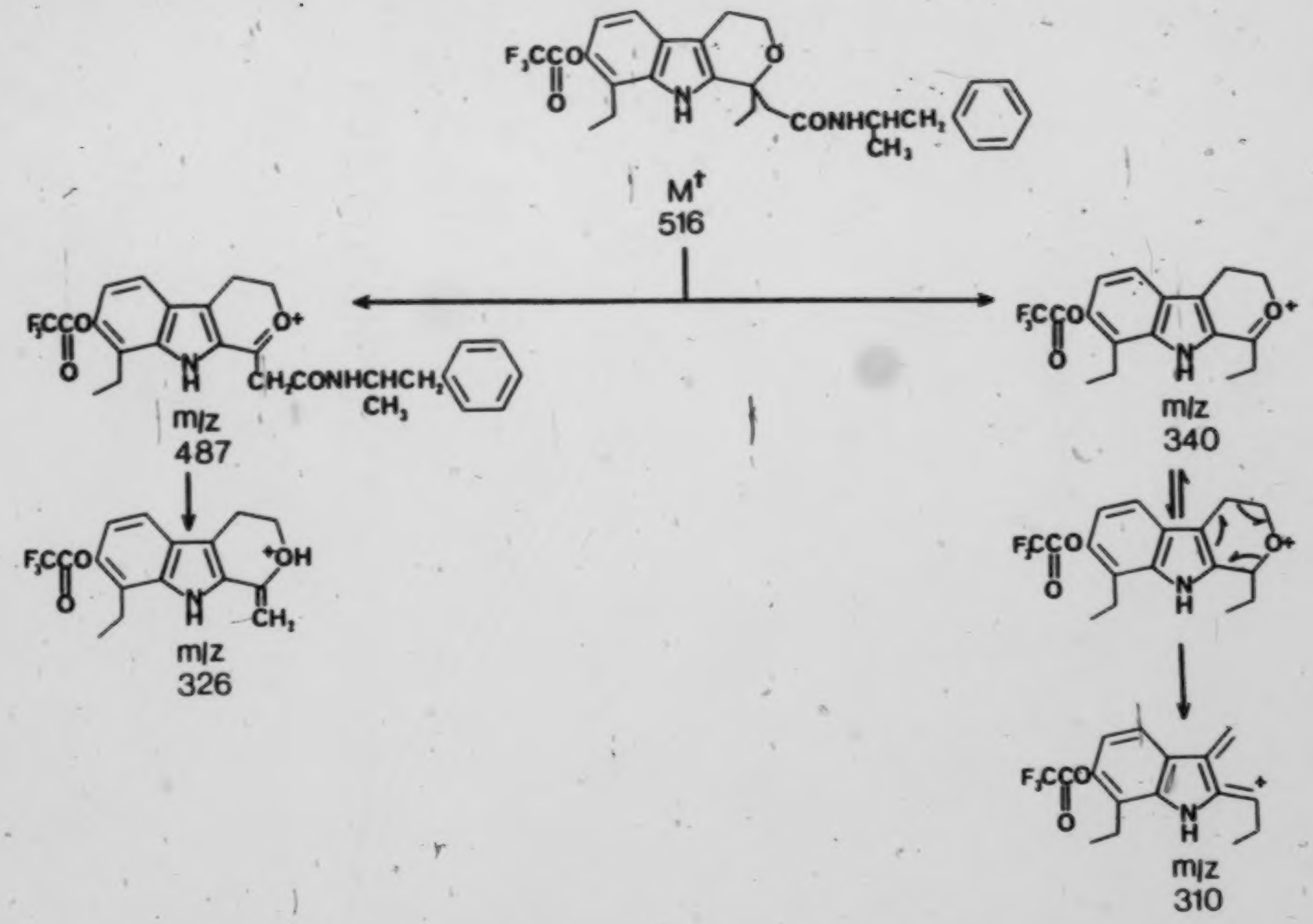

F$_3$CCO
O
N
H
CONHCHCH$_2$
CH$_3$
M$^+$
516
O+
CH$_2$CONHCHCH$_2$
CH$_3$
m/z
487
+OH
CH$_3$
m/z
326
m/z
340
m/z
310

Figure 92. Postulated MS fragments of trifluoroacetylated diastereomeric 7-hydroxyetodolac.

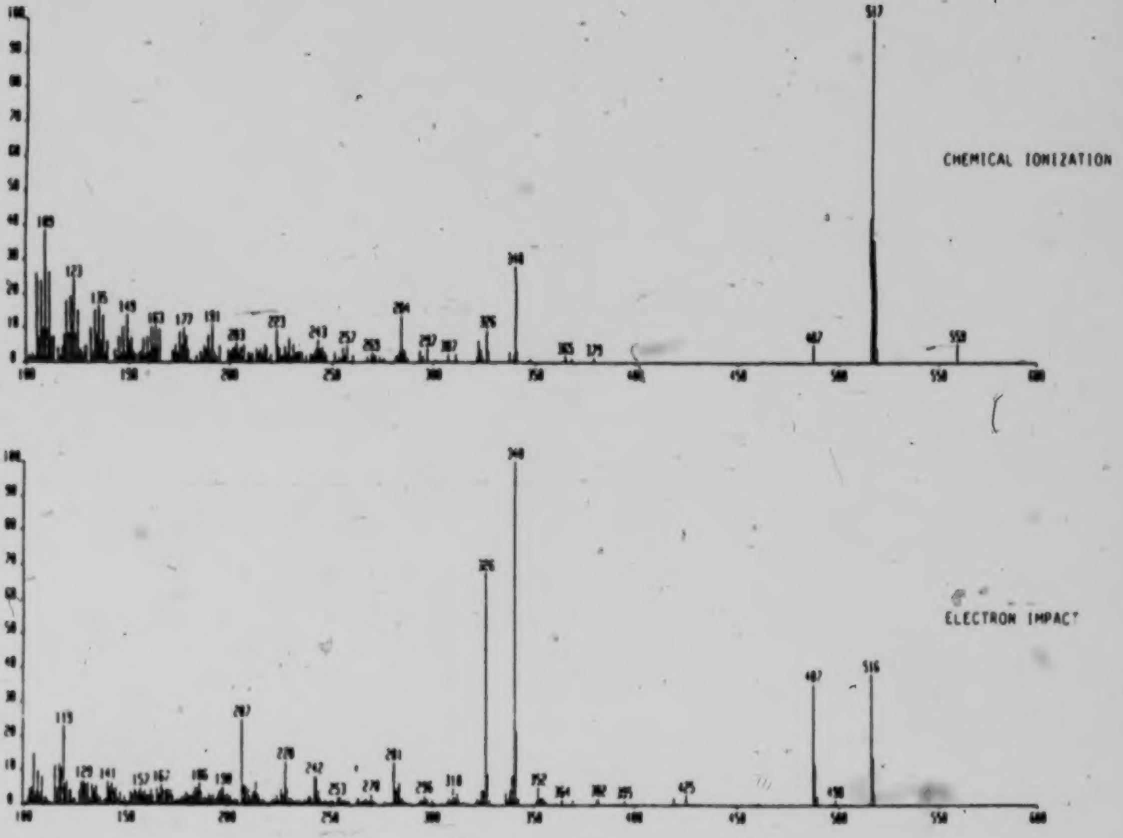


Figure 93. GC-MS line diagrams (CI and EI) of 7-hydroxyetodolac derivatized with S(+)-amphetamine followed by trifluoroacetylation.

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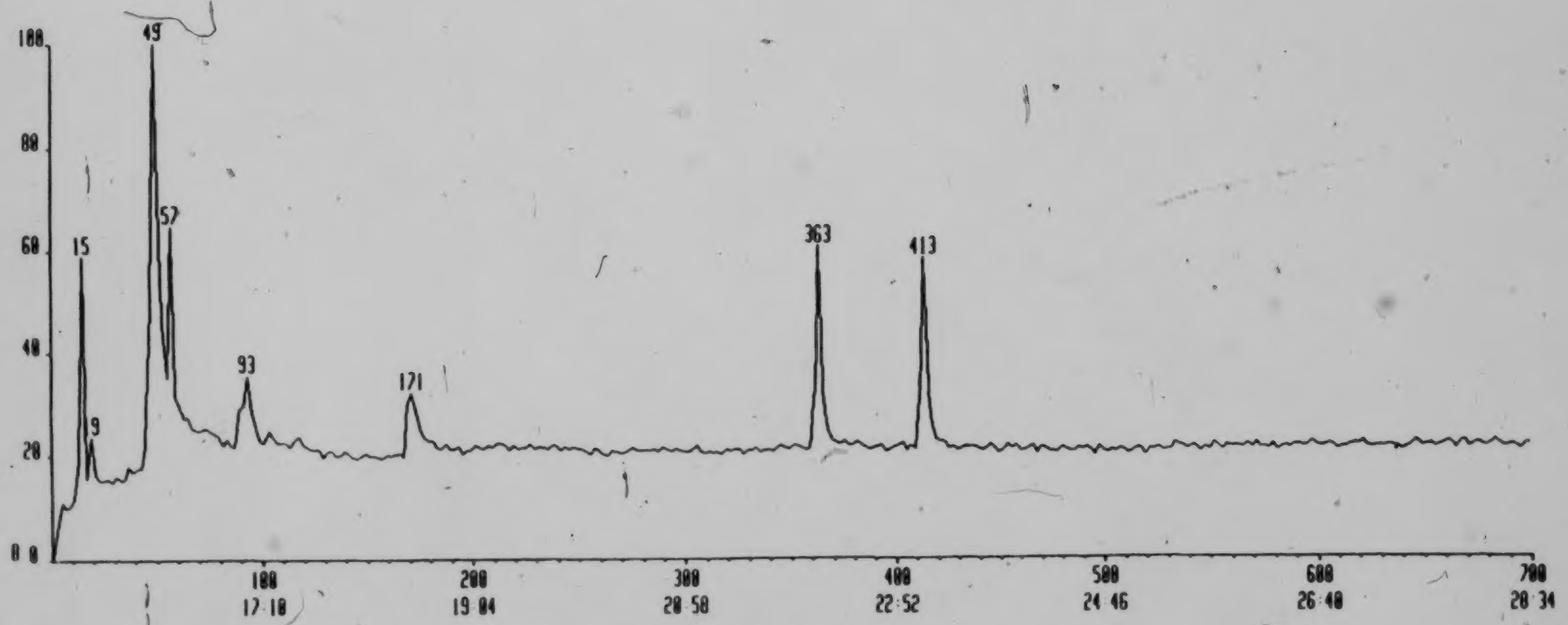

Figure 94. GC-MS total ion trace of 7-hydroxyetodolac derivatized with S(+)-amphetamine followed by trifluoroacetylation (peaks 363 and 413).

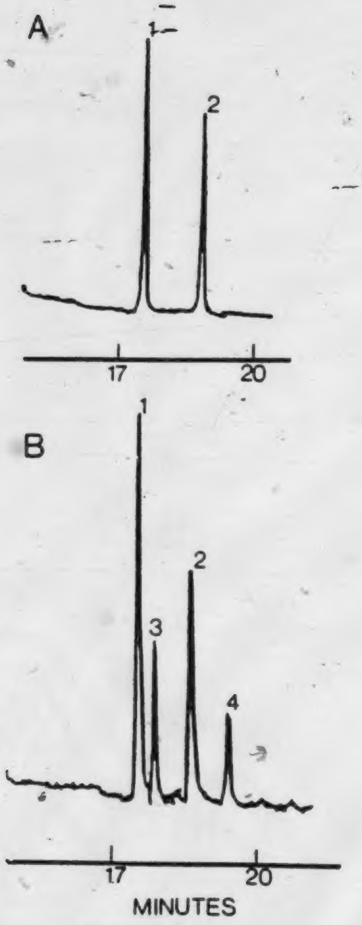

Figure 95. Gas chromatograms of standard etodolac (A) and 7-hydroxyetodolac (B) derivatized with S(+)-amphetamine followed by trifluoroacetylation.

Key: 1 = (+)-etodolac; 2 = (-)-etodolac; 3,4 = 7-hydroxyetodolac isomers.

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APPENDIX 1

The separation factor (α) was calculated using the equation $RT_2 - RT_0/RT_1 - RT_0$; the value of RT_0 was assumed to be 0.

HETP was determined by the equation HETP = L'/n.

The calibration curves were defined by the equation of straight line y=bx+a, where x is the concentration and y is the ratio of peak-areas of the substrate to that of the intrernal standard.

For studies in Section 3.1.0.0.0.0, the values for Kmax and Vmax were calculated using the Lineweaver-Burk equation and KF was determined by the method of residuals (Gibaldi and Perrier, 1982).

Values for Vmax and Kmax for the studies in Section 3.2.0.0.0.0 were determined by the following equations (Gibaldi and Perrier, 1982):

$$\text{Kmax} = \frac{C_0}{2.303 \log \frac{C_{0^*}}{C_0}}$$

Vmax = slope(of concentration-time curve) X Kmax X 2.303

The elimination rate constants and half-lives were determined from the log-linear phase of concentration-time curves using a curve-fitting program (Hewlett-Packard).

AUCs were calculated using the linear trapezoidal method.

As no attempts were made to determine absolute bioavailability in the human studies, CL/F and Vd/F were calculated as indicative of CL and Vd.